Effect of Oral Creatine Supplementation on Urinary Methylamine, Formaldehyde, and Formate

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ABSTRACT

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Purpose: It has been claimed that oral creatine supplementation might have potential cytotoxic effects on healthy consumers by increasing the production of methylamine and formaldehyde. Despite this allegation, there has been no scientific evidence obtained in humans to sustain or disprove such a detrimental effect of this widely used ergogenic substance. **Methods:** Twenty young healthy men ingested 21 g of creatine monohydrate daily for 14 consecutive days. Venous blood samples and 24-h urine were collected before and after the 14th day of supplementation. Creatine and creatinine were analyzed in plasma and urine, and methylamine, formaldehyde, and formate were determined in 24-h urine samples. **Results:** Oral creatine supplementation increased plasma creatine content 7.2-fold ($P < 0.001$) and urine output 141-fold ($P < 0.001$) with no effect on creatinine levels. Twenty-four-hour urine excretion of methylamine and formaldehyde increased, respectively, 9.2-fold ($P = 0.001$) and 4.5-fold ($P = 0.002$) after creatine feeding, with no increase in urinary albumin output (9.78 ± 1.93 mg·24 h^{-1} before, 6.97 ± 1.15 mg·24 h^{-1} creatine feeding). **Conclusion:** This investigation shows that short-term, high-dose oral creatine supplementation enhances the excretion of potential cytotoxic compounds, but does not have any detrimental effects on kidney permeability. This provides indirect evidence of the absence of microangiopathy in renal glomeruli.

Key Words: CREATININE, KIDNEY, ALBUMIN, URINE, CYTOTOXIC COMPOUNDS

Creatine monohydrate supplementation is widely consumed by competitive athletes and sport leisure participants in attempt to enhance performance, delay fatigue, and improve recovery (22). Short-term (3 months) beneficial effects have also been described in patients suffering from myopathies (10). Two cases of renal dysfunction subsequent to creatine intake have been reported (9,17). However, previous studies from our laboratory have found no deleterious health effects on kidney function in healthy humans submitted to short-, medium-, and long-term supplementation of oral exogenous creatine (13–16).

The conversion of creatine to sarcosine results in cytotoxic agents such as methylamine (23). The latter has been found to be deaminated by semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) to produce formaldehyde (26) (Fig. 1). Methylamine and formaldehyde are two well-known cytotoxic agents, the presence of which can be revealed by urine analyses (4,12,25,26).

Based on a review paper by Wyss and Kaddurah-Daouk (23), a French Agency (AFSSA) (1) claimed unequivocally that excess consumption of creatine and creatinine might induce derived carcinogenic and mutagenic compounds that could put athletes and consumers of exogenous creatine at risk (http://www.afssa.fr).

To shed light on those allegations, we investigated the appearance of methylamine, formaldehyde, and formate, the main metabolite of formaldehyde, in urine of healthy humans who received oral creatine supplementation.

METHODS

Twenty healthy male subjects, aged 24.1 ± 1.3 yr (mean ± SEM), volunteered and gave their free consent to the following protocol, which was approved by the medical ethic committee of the Faculty of Medicine (Free University of Brussels). For 14 d, the subjects consumed 21 g of creatine monohydrate (Flamma, Italy) daily, divided into three doses of 7 g each (morning, noon, evening), dissolved in water or fruit juice. The purity of creatine monohydrate has been tested by gas chromatography (99.9%) and found to be devoid of any detectible level of sarcosine as tested by enzymatic reaction. Energy intake was recorded by a food questionnaire over seven continuous days. Most foods were weighed before cooking and eating. Dietary intakes were

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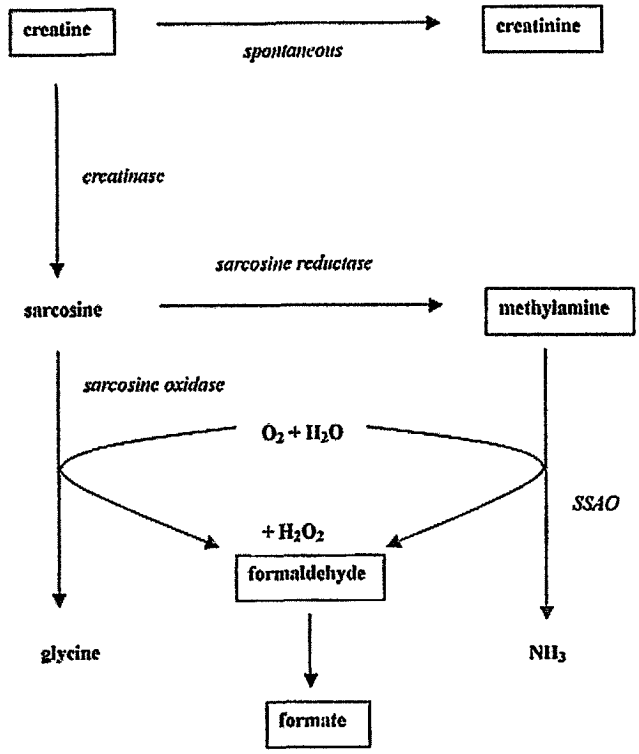

FIGURE 1—Schematic pathways of creatine and creatinine degradation in the human body. All products and metabolic steps are not shown. Compounds that are framed have been assayed in urine before and after creatine supplementation. SSAO, semicarbazide amine oxidase.

assessed by Prodiet 5.1 (Proform, France), professional software that displays the nutrient analysis of any food or combination of selected foods. After that period of food stabilization (mean 3353 kcal, 54% carbohydrate, 31% fat, and 15% protein), the subjects were asked to remain on their usual diet for 2 wk. Twenty-four-hour urine collection was obtained before creatine intake and at the end of creatine supplementation. Venous blood samples were drawn after an overnight fast before creatine feeding and at the end of creatine supplements. All samples (urine and plasma) were stored at −20°C until analyses were done.

Creatine and creatinine were determined in plasma and urine using an enzymatic colorimetric test PAP (Boehringer Mannheim-Roche, Germany). Formaldehyde concentrations in urine were determined by high-performance liquid chromatography by derivatization of 2,4-dinitrophenylhydrazine (4). Formate concentrations in urine were assayed by an enzymatic method using formate dehydrogenase and NAD^+ (6). Urine methylamine was determined by a stable isotope dilution gas chromatography-mass spectrometry method. We developed this from a gas chromatography procedure that used derivatization with isobutyl chloroformate (11). Albumin concentration in urine was determined by an immunochemical technique using specific monoclonal antiserum (Turbiquant, Behring). To allow for urine volume intra- and interindividual variation, urinary values were expressed as the 24-h excretion rate.

When the values followed the Gaussian distribution (Kolmogorov–Smirnov test), the concentration differences before and after creatine feeding were tested by the Student's *t*-test. For data that was not normally distributed, the *t*-test was applied after logarithmic transformation. The results are reported as means and SEM. The statistical significance was fixed at $P \leq 0.05$, two tailed.

RESULTS

Table 1 shows the data obtained on creatine and creatinine concentrations in plasma and urine samples before and after creatine feeding. After an overnight rest following the last intake of creatine, there was a significant increase in venous plasma creatine level (7.2-fold of the basal level) and there was no effect on plasma creatinine level. Creatine urine excretion rates were also highly elevated (141-fold of the basal level) after creatine feeding with no significant changes of the creatinine output. Approximately 77% of the daily dose of ingested creatine was released in the final urine. The albumin urine excretion rate was not affected by creatine supplementation (Table 1).

Table 1 also includes the modifications of urine excretion of formaldehyde, formate, and methylamine before and after creatine supplementation. Twenty-four-hour urine output of methylamine and formaldehyde increased 9.2- and 4.5-fold, respectively ($P < 0.001$), after creatine feeding with no increase in formate excretion. After creatine feeding, there was no correlation between plasma creatine and urine methylamine ($r^2 = 0.025$, P = not significant (NS)) or formaldehyde ($r^2 = 0.017$, P = NS).

DISCUSSION

The results of the present investigation indicate that short-term, high-dose oral creatine ingestion in healthy subjects enhances the mechanisms leading to the conversion of creatine to sarcosine and then to methylamine, the latter one giving rise to formaldehyde. The conversion of formaldehyde to formate should be rather rapid in cells, the latter representing indirectly the production of the former substrate (3). Using rat and mice models, Yu and Deng demonstrated that *in vivo* deamination of methylamine produces formaldehyde and hydrogen peroxyde, which are both recognized as cytotoxic substances (4,24). Moreover, the inhibition of SSAO, the enzyme that converts methylamine into formaldehyde, enhances the urine output of methylamine

TABLE 1. Mean values (± SEM) of plasma and urine contents before and after creatine supplementation.

	Before Creatine	After Creatine
Plasma		
Creatine (μmol·L^{-1})	73.1 ± 7.8	526.9 ± 81.4*
Creatinine (μmol·L^{-1})	97.2 ± 12.5	72.5 ± 10.9
Urine		
Creatine (g·24 h^{-1})	0.11 ± 0.02	15.66 ± 2.06*
Creatinine (g·24 h^{-1})	1.86 ± 0.14	2.22 ± 0.14
Albumin (μg·24 h^{-1})	9.78 ± 1.93	6.97 ± 1.15
Methylamine (mg·24 h^{-1})	0.69 ± 0.06	6.41 ± 1.45*
Formaldehyde (μg·24 h^{-1})	64.78 ± 16.28	290.4 ± 66.3*
Formate (mg·24 h^{-1})	12.46 ± 1.04	14.16 ± 1.84

* $P < 0.001$ between values before and after creatine supplementation.

after creatine feeding in mice (4,24). Consequently, these authors hypothesized that long-term administration of large quantities of creatine as an ergogenic supplement would increase the production of methylamine and subsequently formaldehyde, both being potentially cytotoxic in renal glomeruli (4,24). Our results support this hypothesis in humans.

Despite the 9.2-fold increase in methylamine urine excretion induced by creatine ingestion, this level did not reach the normal upper limit values from healthy humans, up to 35 mg·d^{-1} (mean ± 3 SD) (12). After creatine supplementation, urine formate excretion remained below the upper range (14–20 mg·d^{-1}) reported in healthy subjects (2,7,19). However, with creatine supplementation, the urine excretion of formaldehyde increased 4.5-fold of the basal rate. It has been claimed that formaldehyde is a mutagenic and genotoxic agent that reduces the rate of DNA synthesis (20); however, in human, besides our present results, there are not yet available data on the upper limit of the urinary level of formaldehyde that would indirectly support a mutagenic effect *in vivo*.

Because creatine is transformed to sarcosine by microbial enzymatic reactions (23), it is likely that methylamine is formed in the intestine and is therefore potentially harmful to the integrity of the intestinal epithelium. Methylamine is toxic to human endothelial cells and forms patchlike lesions (27) and even causes kidney damage (26). In mammals, SSAO activity has been found in various tissues associated with the vascular system (5,8). Therefore, it is likely that the deamination of methylamine occurs in the circulation. It could also be speculated that this flooding of methylamine in the blood, together with SSAO, might produce formaldehyde favoring microangiopathy in the renal glomeruli (8,25).

Our subjects consumed a total amount of 294 g creatine monohydrate during 14 d without any modification of glomerular membrane permeability as assessed by their albumin urine excretion rate (9.78 ± 1.93 mg·24 h^{-1} before creatine; 6.97 ± 1.15 mg·24 h^{-1} after creatine). The upper limit in healthy humans is 25 mg·24 h^{-1}. Albuminuria has long been known to be associated with specific renal abnormality and is now recognized as an early test for vascular endothelial damage (18). Despite the fact that formaldehyde and methylamine excretion rates were increased, respectively, 4.5- and 9.2-fold after high-dose of creatine supplementation, there was no detectable consequence of glomerulonephropathy. In this context, it has been shown, at least in rats, that formaldehyde administration in drinking water supplied *ad libitum* during 2 yr can produce specific carcinogenic effects on various organs and tissues (21). This raises the question of the duration of the supplementation. In a previous study, we did not observe any adverse effect of a long-term (up to 5 yr) creatine supplementation in humans (14). Moreover, the present study has only addressed 2-wk supplementation with high-dose creatine (21 g·d^{-1}). The more standard procedure of oral creatine is based on 2–5 g·d^{-1} after an initial 5 d at a higher dose. Thus, there is reason to believe that lower doses of creatine supplementation would reduce the impact on urinary methylamine and formaldehyde excretion. However, even if systematic deleterious effects could not be observed, it cannot be excluded that systematic production of low extra doses of cytotoxic agents never induces any occurrence of nephropathy. Clearly, epidemiological data are required to evaluate the potential risks over a larger cohort of individuals. But in terms of results of the present investigation, caution should be applied. Kidney function of the patients and healthy subjects supplemented with creatine on a regular basis should be systematically monitored throughout the ingestion period.

In conclusion, short-term, high-dose oral creatine supplementation in young healthy subjects increases urinary excretion of methylamine and formaldehyde within the normal range for a healthy population. In the conditions of the current study, increased urinary methylamine and formaldehyde excretion did not result in impaired renal function. Further studies are needed to evaluate whether long-term creatine supplementation, indeed, is harmless in all individuals.

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FNH should stop oral contraceptives.[1-5] However, it is uncertain whether pregnancy should be discouraged or whether surgical treatment is required before or even during pregnancy.[3,5] The few case reports are conflicting. We report 10 women with FNH who became pregnant.

Between 1980 and 1996 191 women presented to our institution in whom FNH was diagnosed. Ultrasound, bolus-enhanced computed tomography, and cholescintigraphy were done. Diagnosis of FNH was made when at least two of the three procedures were unambiguously positive, always including cholescintigraphy. (Patients with equivocal findings and exclusion of haemangioma by labelled red-blood cell scanning or magnetic resonance imaging are considered for surgery because adenoma or even hepatocellular carcinoma cannot be ruled out.) Due to an unclear character of the lesion, tumour-related symptoms, or substantial tumour growth, 109 women had surgery. 82 women with a clear diagnosis of FNH were observed (median age 33 [18–61] years). Routine follow-up included ultrasound every 6 months. Ten of 82 observed women with FNH (12·2%) became pregnant, three patients twice. Median age at the time of tumour diagnosis was 27 (22–32) years. Median tumour size was 10 (4–13) cm. All patients had a history of oral contraception with a median use of 120 (6–168) months. Between the time of diagnosis and delivery a median 33 (2–118) months had gone by. The course of pregnancy was uneventful in 11 of 13 pregnancies. One patient presented with right upper abdominal pain. Another developed vaginal bleeding during the 7th week of pregnancy. No increase in tumour size was observed. Delivery occurred spontaneously in eight cases, by Caesarean section in four, and by forceps in one woman without further problems. All children were alive and healthy. Follow-up after pregnancy and a median of 70 (28–141) months occurred at our institution in seven patients, and in three elsewhere. In eight patients, the tumour size remained the same, and in two a decrease (3 cm and 2·7 cm, respectively) was seen.

During the same period three of 47 (6·4%) women having surgery for hepatocellular adenoma had a complicated pregnancy, before referral to us. They had taken oral contraceptives before getting pregnant. In one case emergency Caesarean had been done during the 34th week for "ablatio placentae" and missing fetal heart sounds. Surgery led to the diagnosis of haemoperitoneum, haematoma, and a tumour in the left lobe of the liver. The second patient had presented with hypovolaemic shock due to a ruptured adenoma 3 days after the expected date of birth. Both patients had still births. The third patient had developed upper abdominal pain 1 week after uneventful caesarean section. Ultrasound and computed tomography revealed intrahepatic bleeding from a liver tumour.

In order to justify observation in patients with FNH a clearcut diagnosis is mandatory, especially since the differential diagnosis between FNH and adenoma may be extremely difficult. In these hypervascularised tumours fine-needle biopsy often has a considerable risk of misdiagnosis and bleeding. Therefore, agreement exists about the need to combine several imaging modalities.[1,4,5] Cholescintigraphy allows visualisation of FNH and highlights the absence of proliferated bile ducts in adenoma. From histological diagnosis of the resection specimen in 62 patients who underwent surgery for solid benign liver tumours, we showed a sensitivity of 82·1%, specificity of 97·1%, positive predictive value of 95·8%, negative predictive value of 84·6%, and accuracy of 90·3%.[4] Therefore, addition of cholescintigraphy to ultrasound and bolus-enhanced computed tomography can lead to a high diagnostic yield, while other authors prefer magnetic resonance imaging.[4] Patients with ambiguous diagnosis should have surgery because hepatocellular adenoma and even carcinoma cannot be ruled out. In cases of observation for FNH, it is reasonable to recommend discontinuation of oral contraceptives until clear data are available. However, there is not enough evidence to advise women against pregnancy or to resect the tumour before pregnancy. Life-threatening rupture during pregnancy may occur in patients with non-operated adenomas, whereas for clearly diagnosed FNH no substantial risks have been found in our cases. It seems to be safe enough to observe tumour size with ultrasound and to favour Caesarean section when there is tumour growth.

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Renal dysfunction accompanying oral creatine supplements

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The use of oral creatine supplements as a legitimate means to augment athletic performance in both professional and amateur sportsmen and women is widespread.[1,2] However, the safety of oral creatine has been questioned with the deaths of three American college wrestlers linked to its use. The dosage regimens suggested by the manufacturers are 20 g daily for 3 to 7 days and then 2 to 5 g daily as a maintenance dose. At these doses there have been no reports of severe side-effects other than a mild increase in plasma creatinine that is reversible on discontinuation of the preparation.[3,4] However, we have recently managed a patient in whom substantial renal dysfunction was apparent.

A 25-year-old man with focal segmental glomerulosclerosis presented, 8 years ago, with frequently relapsing steroid-responsive nephrotic syndrome. He had required treatment with cyclosporin for the past 5 years to minimise nephrotic relapses. Plasma cyclosporin concentrations were maintained at 75–125 ng/mL. During this time renal function was normal with serum creatinine values of 100–110 μmol/L, creatinine clearances of 91–141 mL/min, and an isotopic glomerular filtration rate (GFR) of 122 mL min^{-1} 1·73 m^{-2} (documented July, 1993). He was seen in June, 1997, when his serum creatinine was 103 μmol/L and creatinine clearance 93 mL/min. However, when he attended clinic 12 weeks later the serum creatinine was 159 mmol/L and creatinine clearance 61 mL/min. 1 month later (mid-October, 1997) his renal function had deteriorated further (serum creatinine 180 μmol/L and creatinine clearance 54 mL/min). He had remained in good health with no relapse of proteinuria and he was normotensive with a cyclosporin concentration well within the therapeutic range. He denied taking nephrotoxic medication, but he did admit to taking creatine supplements to augment his pre-season soccer training regime. He had started the preparation in mid-August with a loading dose of

5 g three times per day for 1 week and then a maintenance dose of 2 g/day which he had been taking for 7 weeks at the time of the appointment in October. He was advised to stop the creatine, an urgent isotopic GFR was arranged, and a plan to re-biopsy made should his renal function deteriorate further. The GFR 3 days later was 67 mL min^{-1} 1·73 m^{-2}. 1 month after stopping the supplements his plasma creatinine had returned to 128 μmol/L with a creatinine clearance of 115 mL/min.

There is strong circumstantial evidence that creatine was responsible for the deterioration in renal function in this case. Whether this represents tubular damage due to muscle pigment, or is a manifestation of increased creatine production, is unclear. Physicians should be aware that creatine, which is classed as a food-stuff in the UK, is freely available over the counter and is used at excessive doses by some individuals.

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PI^{A2} polymorphism and efficacy of aspirin

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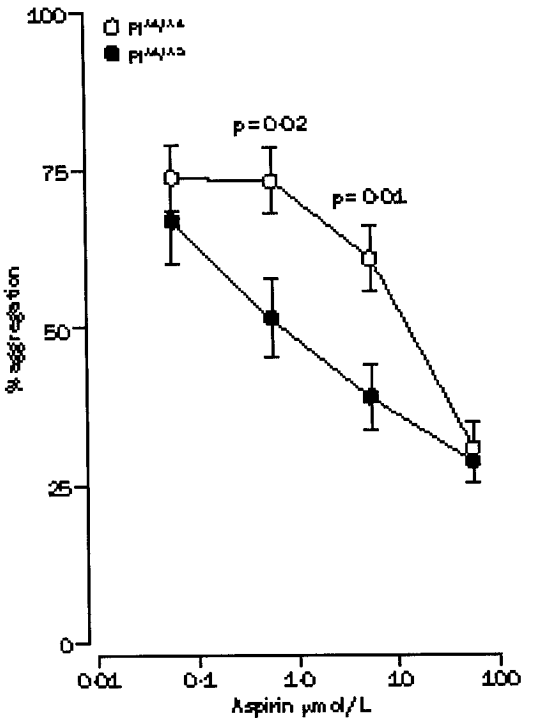
Aspirin is widely used as an antiplatelet drug to prevent arterial thromboembolic events.[1] Aspirin acetylates irreversibly the enzyme cyclooxygenase (COX-1), and thereby inhibits the production of thromboxane A_2 in platelets. However, the mechanism by which thromboxane A_2 induces the expression of high-affinity receptor molecules for fibrinogen on the surface of platelets (the final common pathway of platelet activation) remains unknown.

We have reported that the PI^{A2} polymorphism of the GPIIIa subunit of the fibrinogen receptor (GPIIb-IIIa) is a risk factor for coronary heart disease.[2] Feng et al have shown, on 1336 participants in the Framingham Offspring Study that the PI^{A2} polymorphism increases platelet aggregation in an allele-dependent fashion.[3] While some subsequent studies have found PI^{A2} to be a risk factor for coronary heart disease,[4] others have not, and in particular, the Physicians' Health Study did not show an association between the PI^{A2} polymorphism and first myocardial infarction.[5] We hypothesised that a differential sensitivity to aspirin between PI^{A2}-positive platelets ($PI^{A1/A2}$ or $PI^{A2/A2}$) and $PI^{A1/A1}$ homozygous platelets might explain, at least in part, this discrepancy.

We compared the aggregation of platelets from fifteen $PI^{A1/A1}$ homozygotes and eleven $PI^{A1/A2}$ heterozygotes matched for age, race, and gender. Fasting morning blood was obtained without caffeine intake (>12 h) or drug intake (>7 days). Platelet-rich plasma was obtained from citrated blood and the aspirin-sensitive agonist epinephrine was used to induce aggregation. Adenosine diphosphate (1 μmol/L) was added if epinephrine alone was unable to induce 60% or more aggregation. We tested the inhibitory efficacy of aspirin using aspirin concentrations ranging from 0·053 to 53 μmol/L to provide a spectrum that covers concentrations found in patients treated with various dosages of aspirin (0·6 to 14 μmol/L).



Inhibition of aggregation of $PI^{A1/A1}$ and $PI^{A1/A2}$ platelets by aspirin

Aggregation in the absence of aspirin was the same in $PI^{A1/A1}$ and $PI^{A1/A2}$ platelets. Inhibition of $PI^{A1/A2}$ platelets with aspirin was greater (figure). The mean aspirin concentration that was required to induce 50% inhibition of the aggregation response (IC_{50} in $PI^{A1/A1}$ platelets was 22·8 (5·8 SEM) and 2·3 (1·2) μmol/L in $PI^{A1/A2}$ platelets, indicating a significant (p=0·005) 10-fold, reduction in IC_{50} for aspirin with $PI^{A1/A2}$ platelets.

We conclude that the mechanism of platelet inhibition by aspirin involves an epitope of the GPIIIa subunit of the fibrinogen receptor that contains the PI^{A} polymorphism. We suggest that differences in exposure of patients to aspirin at the time of onset of myocardial infarction might account for some of the discrepancies amongst studies on PI^{A2} as a risk factor for unstable ischaemic coronary events.

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Medicine in focus

Duchenne muscular dystrophy: Focus on pharmaceutical and nutritional interventions

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Abstract

Duchenne muscular dystrophy is a lethal X-linked muscle disease resulting from a defect in the muscle membrane protein dystrophin. The absence of dystrophin leads to muscle membrane fragility, muscle death (necrosis) and eventual replacement of skeletal muscle by fat and fibrous connective tissue. Extensive muscle wasting and respiratory failure results in premature death often by the early 20s. This short review evaluates drug and nutritional interventions designed to reduce the severity of muscular dystrophy, while awaiting the outcome of research into therapies to correct the fundamental gene defect. Combinations of dietary supplementation with amino-acids such as creatine, specific anti-inflammatory drugs and perhaps drugs that target ion channels might have immediate realistic clinical benefits although rigorous research is required to determine optimal combinations of such interventions.

Keywords: Duchenne muscular dystrophy; Mdx mouse; Pharmaceuticals; Nutritional supplements; Therapy

1. Duchenne muscular dystrophy and the mdx mouse model of DMD

There are many forms of muscular dystrophy but only Duchenne muscular dystrophy (DMD) is discussed here. DMD is an X-linked lethal muscle wasting disorder, affecting approximately 1/3500 male births. The disease is caused by a mutation in the gene that encodes for the sub-sarcolemmal protein dystrophin (Biggar, 2006). Dystrophin links the muscle cytoskeleton through a membrane complex to the extracellular matrix. Dystrophic myofibres are susceptible to damage during mechanical contraction, damage leads to myofibre necrosis and ultimately the replacement of myofibres by fibrous and fatty connective tissue (due to failed regeneration). While the genetic defect was identified in 1987, the specific mechanism of myofibre damage is still unclear (Whitehead, Yeung, & Allen, 2006) and there is still no effective treatment for DMD. Therapeutic approaches for DMD fall into three main strategies: (i) replacement of dystrophin by genetic, cell transplantation or molecular interventions; (ii) enhancement of muscle regeneration or reduction of fibrosis to combat failed regeneration; (iii) reduced muscle necrosis. This latter approach is the main focus of this review which outlines pharmacological interventions and nutritional supplementation as potential therapies to reduce myofibre necrosis in DMD. Most experimental studies use mdx mice and therefore data from this

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model, along with clinical studies, form the basis of this review. DMD primarily affects skeletal and cardiac muscle and in addition other tissues (Biggar, 2006), but only the effects on skeletal muscle will be addressed.

The mdx mouse is the most widely used animal model for DMD. The absence of dystrophin results in a distinct disease progression with an acute onset of skeletal muscle necrosis around 3 weeks of age in young mdx mice (Fig. 1), necrosis then decreases significantly after 4–6 weeks to a relatively low level in adult mice (McGeachie, Grounds, Partridge, & Morgan, 1993): the pathology is far more benign than in DMD. The acute onset of myofibre necrosis provides an excellent model to study therapeutic interventions to prevent or reduce necrosis. In contrast, reduced necrosis can be difficult to detect in adult mice where there is little active myofibre breakdown but high cumulative muscle pathology. For this reason, exercise is often used to induce muscle damage

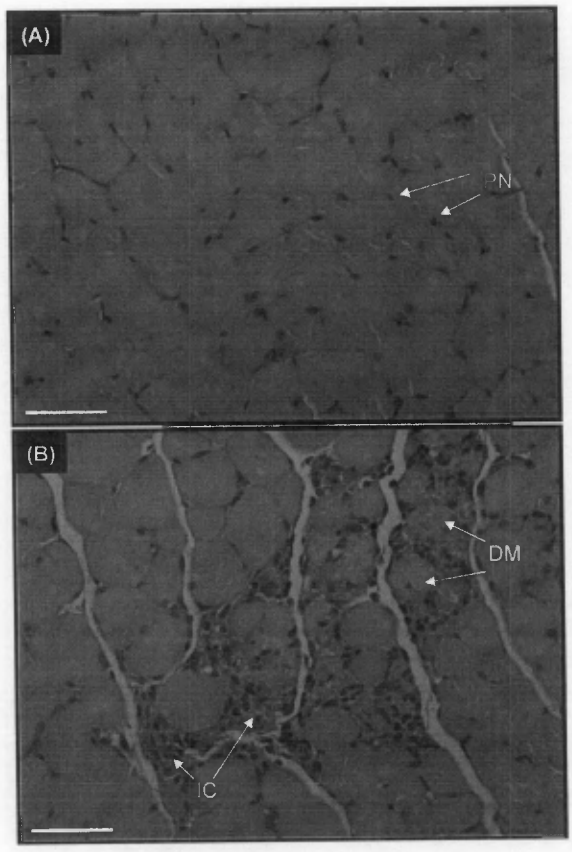

Fig. 1. Skeletal muscle necrosis in the mdx mouse. (A) Pre-necrotic (normal) skeletal muscle in the quadriceps muscle of a 20-day-old mdx mouse, characterized by healthy myofibres with peripheral nuclei (PN). (B) Necrotic skeletal muscle in the quadriceps muscle of a 23-day-old mdx mouse, characterized by infiltrating inflammatory cells (IC) and degenerating myofibres (DM). Transverse muscle sections stained with haematoxylin and eosin. Scale bar represents 50 μm.

enabling potential therapeutic interventions to be evaluated in adult mdx mice (Granchelli, Pollina, & Hudecki, 2000; Payne et al., 2006). The simplest form of exercise is voluntary wheel running, where muscle necrosis in the quadriceps is doubled (from ~6 to 12%) after 48 h (Hodgetts, Radley, Davies, & Grounds, 2006; Radley & Grounds, 2006). Forced exercise greatly increases muscle damage with the most severe injury resulting from forced downhill running (eccentric exercise), although such severe muscle damage caused by eccentric exercise is a poor model for pre-clinical drug screening. The symptoms of dystropathology are cumulative, with fibrosis becoming increasingly pronounced in older (>15 months) mdx mice. Symptoms are most severe in the mdx diaphragm that more closely resembles the severe pathology of DMD (Stedman et al., 1991).

Numerous parameters are measured to assess the in vivo effects of various interventions. In mdx mice, measurements on whole animals are combined with extensive tissue analysis. Physiological parameters such as Rotarod tests (to test motor co-ordination and fatigue resistance) and grip-strength tests (to measure the maximum amount of force an animal applies by grasping) assess changes in muscle endurance, muscle strength and overall functional capacity. Further physiological tests are conducted in vivo and on isolated muscles in situ or in vitro. Blood sampling and serum creatine kinase (CK) levels provide a qualitative indicator of muscle damage. Histological assessment of tissue sections quantifies cumulative muscle necrosis and regeneration, along with leaky myofibres and immunohistochemical staining identifies changes in location and levels of specific proteins. Other measurements include alterations in channel (Ca^{2+} and Cl^{-}) function that contribute (or sensitise) to disrupted calcium homeostasis and to muscle necrosis (De Luca et al., 2003). A positive result with mdx mice can eventually lead to clinical studies in DMD patients. In humans, the main parameters measured are muscle strength, functional tests and CK levels. The Cooperative International Neuromuscular Research Group (CINRG) performs clinical trials on young DMD patients with various compounds, some of which showed positive results in an early screening program on dystrophic mice (Granchelli et al., 2000): while some of these trials have been published, ongoing results are available on http://www.cinrgresearch.org.

2. Steroids and anti-inflammatory drugs

Until a cure for DMD is found, treatment will involve the administration of corticosteroids combined with interventions to alleviate cardiac and respiratory prob-

lems. Corticosteroids have a catabolic effect on muscle (non-exercised muscle) and act to preserve existing muscle fibres and reduce inflammation, although their exact mechanism of action in dystrophic skeletal muscle is unknown. The two main corticosteroids used to treat DMD, Prednisone and Deflazacort, both seem equally effective in delaying the progression of muscle wasting. Unfortunately both are associated with adverse side-effects although these, particularly weight gain, are less severe with Deflazacort. Side-effects of Deflazacort include an increase in appetite requiring strict dietary control, retarded growth resulting in short stature and asymptomatic cataracts. It is recommended that daily calcium and Vitamin D supplementation is taken in conjunction with corticosteroids to maintain bone mineral density and reduce bone fractures (Biggar, 2006). The administration of steroids is not a cure for DMD but a therapy to improve quality of life and prolong lifespan.

DMD is characterized by aggressive inflammation and there is strong evidence that this contributes to myofibre necrosis both in vitro and in vivo (reviewed in Refs. (Tidball & Wehling-Henricks, 2005)). Other immunosuppressive drugs have demonstrated benefits in mdx mice, leading to increasing recognition for the damaging role of inflammation in DMD. Early clinical trials with the immunosuppressive drug cyclosporine in DMD returned promising results as 8 weeks of treatment (5 mg/kg/day) resulted in a significant increase in muscle force generation (Sharma, Mynhier, & Miller, 1993) and cyclosporine reduced the dystropathology in mdx mice (De Luca et al., 2005). However, cyclosporine exerts multiple dose-dependent effects and a correct dosage must be established especially when administered to young dystrophic patients during muscle development. These results are of clinical relevance, as immunosuppression may be required for enhancing efficiency of future gene/cell therapies.

Another potential anti-inflammatory drug that has attracted attention is pentoxifylline. Pentoxifylline has a wide range of anti-inflammatory and anti-coagulant effects; it reduces TNFα production in vitro (Vary et al., 1999), reduces fibrosis and may also play a role in normalising blood flow in dystrophic muscle. On the basis of increased muscle strength in exercised adult mdx mice (Granchelli et al., 2000), pentoxifylline is now the subject of two CINRG trials in DMD patients (one completed recently). However, a recent study in mdx mice involving pentoxifylline (16 mg/kg/day) administration for 4 weeks failed to reduce fibrosis or improve the contractile force of the diaphragm muscle (Gosselin & Williams, 2006), this study does not support the use of pentoxifylline as an anti-fibrotic drug in DMD patients. However, pentoxifylline counteracts, both in vitro and in vivo, the abnormal activity of calcium channels responsible for high sarcolemmal calcium permeability of dystrophic myofibres, suggesting a possible amelioration of dystrophic condition through alternative pathways (Rolland et al., 2006). Both cyclosporine and pentoxifylline (like corticosteroids) affect many cellular events and can have severe adverse side-effects, careful animal studies will be helpful in this regard.

Other anti-inflammatory drugs such as oxatomide (Granchelli et al., 2000) and cromolyn (Granchelli, Avosso, Hudecki, & Pollina, 1996; Radley & Grounds, 2006) that block mast cell degranulation are used widely for clinical treatment of allergies such as asthma and show benefits in mdx mice, indicating that mast cell products (including TNFα) have detrimental effects in dystrophic muscle. A recent CINRG trial with oxatomide (based on the study by (Granchelli et al., 2000)) in DMD showed some minor benefits.

3. Specific anti-cytokine drugs

Another promising approach involves targeting specific aspects of the inflammatory response (rather than the broadly acting anti-inflammatory drugs) in order to reduce muscle necrosis. While systemic depletion of specific inflammatory cells may not be clinically viable, modulation of specific cytokines has been very successful clinically in several severe inflammatory disorders. tumour necrosis factor-alpha (TNFα) is a key pro-inflammatory cytokine that stimulates the inflammatory response and pharmacological blockade of TNFα activity with the neutralising antibody infliximab (Remicade) is highly effective clinically at reducing symptoms of inflammatory diseases such as rheumatoid arthritis and Crohn's disease (Feldman & Maini, 2003). Similar successful clinical blockade of TNFα by the drug etanercept (Enbrel) results from the use of soluble receptors to TNFα. In mdx mice, infliximab delays and reduces the necrosis of dystrophic muscle in young mdx mice (Grounds & Torrisi, 2004). A protective effect of TNFα blockade is reinforced by two recent studies using etanercept that clearly reduces muscle necrosis in young mdx mice (Hodgetts et al., 2006; Pierno et al., 2006) and in exercised adult mdx mice (Hodgetts et al., 2006) with additional physiological benefits on muscle strength, chloride channel function and reduced CK levels being demonstrated in chronically treated exercised adult mdx mice (Pierno et al., 2006). Such emerging highly specific anti-inflammatory drugs designed for use in other clinical conditions, appear an attractive alternative (to steroids) for DMD, although their potential to reduce

the severity of DMD remains to be determined. It may be possible to limit the use of these drugs to periods of intensive muscle growth in boys when muscle damage and deterioration can be especially pronounced. Patients undergoing long-term anti-cytokine treatment must be monitored carefully for serious infections, as is the case for any immunosuppressive drug.

4. Other pharmaceutical interventions

Antioxidants. It is widely recognized that high levels of reactive oxygen species can damage tissues, including skeletal muscle (Rando, 2002). Antioxidants that reduce oxidative damage in cells, such as Coenzyme Q10 (CoQ10) and green tea extract [(−)-epigallocatechin gallate] are the subject of recent research in mdx mice and DMD patients. Green tea extract supplemented diets fed to mdx mice (from birth), significantly reduced muscle damage (necrosis and regeneration) in the EDL muscle of 4-week-old mice and improved muscle function in 8-week-old mice after 5 weeks of treatment (Buetler, Renard, Offord, Schneider, & Ruegg, 2002; Dorchies et al., 2006). CoQ10 is essential for several enzymatic steps in the production of energy and functions as an antioxidant. CoQ10 was the subject of a CINRG pilot study in DMD patients to assess the effectiveness and safety in combination with steroid treatment. CoQ10 increased strength in some muscle groups and a larger follow-up study was recommended. This larger study that is currently being conducted by CINRG, is a 13-month, prospective, randomized study comparing daily Prednisone treatment (0.75 mg/kg/day), CoQ10 (>2.5 μg/mL) and a combined treatment (Prednisone and CoQ10) in older non-ambulatory patients.

4.1. Anabolic effects of β_2-agonist drugs

Anabolic agents result in a net increase in protein content and muscle size and this is usually (but not always) associated with increased strength. Although commonly recognized as asthma drugs, high doses of some β_2-agonists have anabolic effects on muscle and thus the potential to slow muscle degeneration. A 3-month pilot trial of the β_2-agonist albuterol given to patients with fascioscapulohumeral disease improved maximum voluntary strength. This was followed by a year long trial where patients were treated with up to 16 mg of albuterol twice daily resulting in improved muscle mass and grip strength. Albuterol administered for 28 weeks (Fowler, Graves, Wetzel, & Spencer, 2004) to boys with DMD produced a modest increase in strength with no reported side-effects. It is noted that studies with β_2-agonists in mdx mice have returned inconsistent results (Dupont-Versteegden, Katz, & McCarter, 1995; Lynch, Hinkle, & Faulkner, 2000) and that β_2-agonists are associated with numerous undesirable side-effects including increased heart rate and tremors, which have limited their therapeutic potential. More recently synthesized β-agonists such as formoterol, have anabolic effects on skeletal muscle with minimal cardiac side-effects (Ryall, Sillence, & Lynch, 2006) when administered at micro-molar doses in mice and thus may offer a greater potential for DMD (Harcourt, Schertzer, Ryall, & Lynch, 2006).

4.2. Disturbed ion channels and drugs to inhibit proteases

Damaged muscle membranes disturb the passage of calcium ions into the myofibre, and disrupted calcium homeostasis activates many enzymes, e.g. proteases, that cause additional damage and muscle necrosis. Ion channels that directly contribute to the pathological accumulation of calcium in dystrophic muscle are potential targets for drugs to treat DMD. There is evidence that some drugs, such as pentoxifylline, block exercise-sensitive calcium channels (Rolland et al., 2006) and antibiotics that block stretch activated channels reduce myofibre necrosis in mdx mice and CK levels in DMD boys (Whitehead et al., 2006). Calpains are calcium activated proteases that are increased in dystrophic muscle and may directly account for myofibre degeneration (Spencer, Croall, & Tidball, 1995). A new compound, BN 82270 (Ipsen) that has dual action as both a calpain inhibitor and an antioxidant (targeting both calpain and ROS induced muscle damage) increased muscle strength, decreased serum CK and reduced fibrosis of the mdx diaphragm, suggesting a potential therapeutic effect with this new compound (Burdi et al., 2006). A promising new compound of Leupeptin/Carnitine (Myodur) has recently been proposed for clinical trials in DMD patients.

Beyond the diverse approaches mentioned already, there has been much research into substances to help reduce the dystropathology via maintenance or improvement of myofibre size, strength and function. However, strategies such as increasing IGF-1 or other growth factors, inhibition of myostatin, normalising nitric oxide production and the use of poloxamer (P188), do not readily translate into the clinical situation at present. Although these interventions (and many others) have shown promising effects in mdx mice, adverse or unknown long-term systemic effects currently limit their therapeutic potential.

Chinese herbal medicine is becoming increasingly popular as an alternative approach to reduce the severity of symptoms associated with many diseases. For example, ginseng has a diverse range of effects in vivo and a study that showed a reduction in exercise-induced damage in normal muscle after ginseng supplementation (Hsu, Ho, Lin, Su, & Hsu, 2005) suggests possible benefits for DMD. A review of traditional Chinese medicines, such as massage, acupuncture and capsules (that contained herbs and other ingredients) that claimed to alleviate symptoms in DMD patients, was conducted in Beijing in 2003 (Urtizberea, Fan, Vroom, Recan, & Kaplan, 2003). Due to the small number of cases no definitive conclusions could be drawn from this study, although an overall mild frequency of contractures was noted and related possibly to the positive influence of acupuncture and massage. A follow-up study confirmed that high levels of glucocorticoids were present in the capsules and may account for the anecdotal improvement seen in patients (Courdier-Fruh, Barman, Wettstein, & Meier, 2003). Chinese herbal medicine was also found to improve locomotor activity in mdx mice (Chen, 2001). Traditional Chinese medicine is not formally regulated which potentially creates a large risk for incorrect dosing and dangerous content as medicines may unknowingly contain various heavy metals, herbicides, drugs, pesticides and micro-organisms. Furthermore, adverse drug interactions with cumulative effects may result when administered to DMD boys already receiving steroid treatment.

5. Nutritional interventions

Deficiencies of many substances (Selenium, Vitamin E or Vitamin D) can cause severe myopathies, suggesting the importance of nutritional supplementation to counteract these deficiencies: however it seems difficult to justify the application of such supplements to situations of adequate diet or DMD. Muscle wasting is associated with changes in the biochemistry of skeletal muscle that lead to reduced protein synthesis, increased protein breakdown (catabolism) and increased oxidative cell damage. The use of protein powders and specific amino acid supplements has been proposed for attenuating muscle protein loss and to provide a favourable environment for increasing protein synthesis and muscle mass and has received much attention in sports medicine and ageing. Dietary supplementation is a form of protective therapy potentially available for immediate use and broadly falls into three categories; antioxidants or Chinese herbs (both discussed above) and amino acid supplementation.

5.1. Amino acids

Amino acids such as creatine, taurine, glutamine and L-arginine have all been trialed in the mdx mouse with some benefits on muscle strength or dystropathology. Creatine is directly involved in the energy supply of muscle cells and supplementation into the diet (10%, w/w, in chow) of both exercised adult mdx mice and pregnant mdx mothers increased strength and improved dystropathology (De Luca et al., 2003; Passaquin et al., 2002). Similar benefits were seen after intraperitoneal injection of 10mg/kg in exercised adult mice (Granchelli et al., 2000). A double-blinded randomized creatine monohydrate (0.10 g/kg/day) trial in boys with DMD for 4 months showed increased hand grip strength and fat-free mass, independent of steroid usage (Tarnopolsky et al., 2004). A more recent clinical trial conducted by CINRG tested two amino acids (creatine 5g/day and glutamine 0.6 g/kg/day) in DMD boys aged 4–10 years (grouped as 4–7 years and 7–10 years). Although it did not significantly improve muscle strength, creatine was well tolerated by all patients and there was a trend towards less deterioration in other outcomes (Escolar et al., 2005). Creatine monohydrate supplementation in DMD is the subject of a recent review (Pearlman & Fielding, 2006) that concludes that it should be considered as a therapeutic agent for DMD, due to the potential for increased fat-free mass and increased muscle strength. However, additional long-term studies are required to elucidate the role of creatine in skeletal muscle growth and to accurately assess the degree to which creatine exerts protective musculoskeletal effects, without unwanted side-effects such as weight gain and kidney problems.

Taurine is a free amino acid abundant in skeletal muscle that exerts a wide spectrum of actions, ranging from osmolyte control, antioxidant action and anti-inflammatory effects. In skeletal muscle, taurine modulates ion channel function and calcium homeostasis (Conte Camerino et al., 2004). Supplementation of taurine (10%, w/w, in chow) is relatively safe and counteracts exercise-induced weakness after chronic exercise and ameliorates gCL (macroscopic chloride conductance, an index of degeneration-regeneration) in EDL muscles of mdx mice (De Luca et al., 2003). Clinically, taurine has been used with varying degrees of success in a wide variety of conditions (Birdsall, 1998) and is present in commercial food and beverages claimed to work as energizers.

Screening of numerous amino acids for potential efficacy in the mdx mouse (Granchelli et al., 2000) found that intraperitoneal injections of glutamine (10 mg/kg)

Table 1
Summary of potential pharmaceutical and nutritional interventions as therapeutic agents in (mdx mice and) DMD patients

Substance	Action	Mdx mice	DMD patients (+/− benefit)	Immediate clinical potential
Cyclosporine	Immunosuppressant, anti-inflammatory	Maintained muscle strength, cellular parameters & CK levels in exercised adult mice (De Luca et al., 2005)	Eight-week trial—increased muscle force generation (Sharma et al., 1993)	May be (dosage to be carefully decided due to potential toxicity)
Pentoxifylline	Anti-inflammatory, anti-coagulant, anti-fibrotic	Increased muscle strength in exercised mice (Granchelli et al., 2000; Rolland et al., 2006). No improvement in mdx diaphragm fibrosis (Gosselin & Williams, 2006) amelioration of calcium homeostasis (Rolland et al., 2006).	2×CINRG recent clinical trials (one trial on-going)	May be
Oxatomide	Anti-inflammatory histamine (H1) receptor antagonist (asthma therapy)	Increased muscle strength in exercised mice (Granchelli et al., 2000)	Recently completed CINRG clinical trial	Yes
Cromolyn	Anti-inflammatory mast cell stabilizer (asthma therapy)	Increased strength and reduce muscle necrosis in young and adult mice (Radley & Grounds, 2006)	–	Yes
Infliximab (Remicade)	Anti-inflammatory TNFα antibody (arthritis, Crohn's disease therapy)	Reduced muscle necrosis in young mice (Grounds & Torrisi, 2004)	–	Yes (Monitor for possible infections)
Etanercept (Enbrel)	Anti-inflammatory TNFα antibody (arthritis, Crohn's disease therapy)	Reduced muscle necrosis in young and exercised adult mice (Hodgetts et al., 2006; Pierno et al., 2006). Maintained muscle strength, cellular parameters & CK levels in exercised adult mice (Pierno et al., 2006).	–	Yes (monitor for possible infections)
Coenzyme Q10	Antioxidant energy production	–	2× CINRG recent clinical trials (one trial on-going)	Yes
Green tea extract	Antioxidant	Reduced necrosis and improve muscle function in mice (Buetler et al., 2002; Dorchies et al., 2006)	–	Yes (necessary dosage still to be determined)
Chinese herbal medicine	Antioxidant	Improved locomotor activity in adult mdx mice (Chen, 2001)	Anecdotal improvement in patients (Urtizberea et al., 2003) Steroid content confirmed (Courdier-Fruh et al., 2003)	No (exact composition of tablets unknown)
Old β-agonists: clenbuterol, albuterol	Anabolic effects on muscle. Possible anti-inflammatory (asthma therapy)	Inconsistent results in mdx mice (Dupont-Versteegden et al., 1995; Lynch et al., 2000)	Albuterol, 28 week trial resulted in strength increase (Fowler et al., 2004)	May be (potential for cardiac problems)
New β-agonists: formoterol	Anabolic effects on muscle. Possible anti-inflammatory	Improved muscle function in mdx mice (Harcourt et al., 2006)	–	May be/yes (minimal cardiac side-effects)

Table 1 (*Continued*)

Substance	Action	Mdx mice	DMD patients (+/− benefit)	Immediate clinical potential
Calpain inhibitors: BN 82270, Myodur	Inhibit calcium dependent enzymes with additional pharmacodynamic or pharmacokinetic properties	BN 82270, increased muscle strength, decrease CK and muscle fibrosis in mice (Burdi et al., 2006)	Myodur proposed for clinical trials in 2006.	May be (pre-clinical toxicological studies are required)
Creatine	Amino acid (directly involved in muscle metabolism)	Increased strength and improved dystropathology in mice (De Luca et al., 2003; Granchelli et al., 2000; Passaquin et al., 2002)	Four-month trial—increased grip strength and decrease fat mass (Tarnopolsky et al., 2004) 6 month CINRG clinical trial—reduced deterioration of strength (Escolar et al., 2005)	Yes (upon monitoring of possible side-effects)
Taurine	Amino acid (control of calcium handling in vitro)	Maintained strength in exercised adult mice (De Luca et al., 2003)	–	Yes (upon monitoring of possible side-effects)
Glutamine	Amino acid	Maintained strength in exercised adult mice (Granchelli et al., 2000)	CINRG clinical trial (Escolar et al., 2005) 10-day trial—reduced whole-body protein degradation (Mok et al., 2006)	Yes (upon monitoring of possible side-effects)
L-Arginine	Amino acid (substrate for NOS)	Upregulation of utrophin, improved dystropathology and reduced exercise induced muscle damage (Archer et al., 2006; Voisin et al., 2005)	–	Yes (upon monitoring of possible side-effects)
Chinese massage or acupuncture	Stimulation of skeletal muscles and joints	–	Anecdotal improvements in patients (Urtizberea et al., 2003)	Yes

Note: Even though some substances are shown as having the potential for immediate clinical intervention, the issue of adverse side-effects requires careful evaluation and, importantly, some of these substances when administered alone show only marginal benefits.

and a glutamine and alanine combination (10 mg/kg each) significantly improved whole body strength after 6 weeks of treadmill exercise. Clinical studies with glutamine show that oral supplementation (0.5 g/kg/day) over 10 days also inhibits whole-body protein degradation in DMD patients (Mok et al., 2006). Combinations of multiple dietary supplements (including creatine), both with and without prednisolone have recently shown improved muscle strength and reduced fatigue in exercised mdx mice (Payne et al., 2006). Such studies build a good case for promising benefits from combined interventions.

An additional possible treatment for DMD would be to compensate for the loss of dystrophin and nitric oxide (NO) with pharmacological agents. Administration of L-arginine (the substrate for nitric oxide synthase) increases NO production and up regulates utrophin expression in mdx mice. Six weeks of L-arginine treatment (200 mg/kg – intraperitoneal injection) improved muscle dystropathology and decreased serum CK in mdx mice (Voisin et al., 2005) and when given in combination with Deflazacort (Archer, Vargas, & Anderson, 2006) L-arginine (0.375% in drinking water) spared limb muscles from exercise induced damage and increased the distance (km) run voluntarily by an individual mouse. Since L-arginine can have adverse side effects, the drug isosorbide dinitrate that increases NO might be preferable for NO-based therapy in muscular dystrophy (Marques, Luz, Minatel, & Neto, 2005).

While amino acids have been proposed for many clinical conditions, possible benefits to DMD of supplementation with taurine, glutamine, alanine and arginine (alone or in combinations) remain to be formally evaluated.

6. Conclusion

The protective interventions briefly outlined in this review are those with some immediate possibility for clinical application in the near future; either drugs already in clinical use for other purposes or nutritional supplements as summarized in Table 1. When developing therapies for DMD, the goal is to maintain or promote skeletal muscle mass and function but at the same time reduce any deleterious side-effects, such as unwanted cardiovascular complications seen with powerful muscle anabolic agents. Early treatments administered before the pathology manifests are expected to be the most efficacious. Aggressive inflammation is a secondary characteristic of the disease and specific anti-inflammatory drugs seem to be a logical progression in the search for an alternative to steroids. Dietary supplementation in conjunction with some anti-inflammatory drug seems particularly promising and requires further rigorous investigation.

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Does NO metabolism play a role in the effects of vegetables in health? Nitric oxide formation via the reduction of nitrites and nitrates

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SUMMARY

A recent attribution of vegetables to human health stems from their effect on the gasotransmitter nitric oxide (NO). This commentary proposes that a major reason for this attribution is that vegetables are rich in nitrates. Recent research has shown that nitrites and nitrates are not only inert end-products of NO oxidation. In addition, they can be recycled back to bioactive NO and this pathway is an important alternative to the classical L-arginine-NO-synthase pathway.

Various chronic ailments, e.g., diabetes, obesity, high blood pressure, are symptomatic of NO bioavailability. Because NO deficiency is metabolically complex, natural nitrate-rich nutrients, like green leafy vegetables, can improve these chronic ailments via the alternative nitrate–NO pathway.

This commentary implies an added value for vegetables in enhancing health such as cardiac health and in lowering the risks of maladies such as diabetes.

Introduction

Fruit and vegetable supplementation has been associated with lower risk of chronic diseases including cancer and cardiovascular disease and provide beneficial effects that can be attributed to a variety of protective agents [1–3]. In this commentary, the therapeutic attributes of vegetables will be discussed mainly in terms of their nitrates and nitrites content as a source for nitric oxide (NO) [4–7].

The past decade has witnessed a significant increase in the interest of biologists in NO. This highly reactive free radical, first considered only a noxious air pollutant, plays a vital role in many biological events including regulation of blood flow, immunity and neurotransmission [8].

Recent data demonstrate that NO is not only a paracrine substance. It has been shown that enzymatic generation of NO in the heart is capable of modulating remote physiological actions and cell signaling [9]. NO was also shown to affect the computational ability of the brain; it regulates information transmission across neurons [10]. Extensive data from studies on NO signals, on relaxation/stress processes and on health, supports a view of an NO net, serving as a body coordinator [11,12].

The necessity of NO for body functions is suggested by the fact that its deficiency is correlated with various chronic ailments like, obesity [13], diabetes [14], hypertension [15], pulmonary hypertension [16], osteoporosis [17] and old age [18]. Supplementing NO levels is helpful and thus 5-phosphodiesterase inhibitors (e.g., Viagra), have a potential protective role in chronic ailments as coronary artery disease [19,20]. It is also not surprising that though helpful, the response rate to NO related drugs as Viagra is lower in diabetics [21].

The problematic nature of chronic ailments is the occurrence of an extended, not necessarily hazardous, condition (see the following elaboration on obesity). Thus, while temporary deficiency of NO may not pose a dramatic risk, a long term NO deficiency becomes hazardous.

NO bioavailability in the body depends on the rate of its synthesis and metabolism. Though arginine is a main precursor for the NO synthesis [22], it has lately been established that mammalian nitrate and nitrite reduction can also contribute to NO biogenesis [23,24]. This commentary recommends an increase in consumption of nitrate-rich vegetables to prevent NO shortage and its consequential ailments. These nitrate-rich nutrients support health via reduction of nitrites and nitrates to NO [25]. Though beyond the scope of this commentary, it is worth mentioning that vegetable consumption has many nutritional benefits, these include supplementing precursors not only to NO but also to other gasotransmitters such as H2S [26,27].

Discussion

This section is composed of three parts:

- Description of a chronic ailment (obesity) and its NO deficiency.
- Curing NO shortage via nitrates and nitrites supplementation.
- Nitrate-rich vegetables indeed improve chronic ailments.

Abbreviations: NO, nitric oxide; IMT, intima-media thickness.

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Obesity and NO deficiency

Obesity, which begins as simple overweight, over time becomes a malady correlated with ailments such as heart disease and diabetes [28]. NO deficiency may explain this pattern [29]. There are a number of studies that support this idea.

First, NO and citrulline are decreased in obese juveniles when compared to normal weight juveniles, and are negatively correlated with body weight. Arginine (the NO precursor) however, is increased in obese juveniles and is positively correlated with body weight [13]. Therefore, whereas low levels of NO are correlated with obesity, it is not the lack of arginine that limits NO bioavailability.

Second, another study demonstrated that excess of fat in the body results in excess of the leptin hormone, which in turn, can lower the levels of bioavailable NO [30].

It is interesting to note that caloric restriction, without malnutrition, extends life span in a range of organisms [31] and that there is an NO link between caloric restriction and mitochondria [32].

Until the last century, obesity was a rare and impermanent condition and a transient lowered NO communication capacity was not necessarily unhealthy. Today, when obesity is mostly irreversible, prolonged inhibited NO bioavailability is hazardous and can account for the numerous maladies associated with obesity [28].

The reduction of nitrites and nitrates

Increasing the sources of NO, may therefore, contribute to the health of the obese even in the absence of losing weight. When arginine oxidation to NO is prohibited, alternative routes for NO production are effective – the reduction of nitrites and nitrates [33].

Nitrate and nitrite are important alternative sources of NO in especially, but not exclusively, hypoxic states [34]. Following are some examples; The intermittent hypoxia improved glucose tolerance [35]. Nitrite therapy augments ischemia-induced angiogenesis [36], nitrate reduction is emerging as a regulator of physiological functions and tissue responses to myocardial infarction or stroke [23] and brief elevations in plasma nitrite trigger a concerted cardioprotective response [37]. In addition to supplementation of NO compounds in acute situations, it is also effective in improving the urogenital system and skeletal health [38].

As suggested by Tamaki [39], dietary nitrite-derived NO generation may serve as a backup system when the NO synthase/L-arginine-dependent NO generation system is compromised.

Best nitrite and nitrate food sources

Some of the best nitrate-rich foods are lettuce, spinach, beetroot [6] and fruits such as pomegranates [40,41]. Accumulating data shows that these foods improve various chronic ailments. Following are some examples; Beetroot reduces high blood pressure [42], green leafy vegetable consumption is linked to lower risk for diabetes in women [3], nitrate-rich vegetables were shown to decrease the oxygen demand during exercise [43], pomegranate juice consumption by patients with carotid artery stenosis decreases carotid IMT and systolic blood pressure [44] and indeed NO produced from nitrite plays important roles in limiting post-ischemic tissue injury [45].

Because increasing substantially the daily consumption of vegetables is not simple [46–48], it seems worthwhile to spread the trend of drinking vegetable juices [49] when dealing with chronic ailments.

It is interesting to note that the Dunhuang scrolls dating to approximately 800 AD, suggested that nitrite and nitrate were used by the Chinese to relieve chest pain and reduce cold in the hands [50].

Conclusions

Regulation of NO function is showing itself to be a complex event that maladies are associated with its disruption.

Here, it is proposed that vegetables can at least partly, reverse these effects by increasing NO generation via nitrate and nitrite. This explains how encouragement for daily consumption of extra vegetables supports good health.

Vegetables can be considered a natural "drug" with sustained release of low-dose nitrite into the circulatory system, alternative source to the L-arginine pathway to NO.

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EFFECTS OF CREATINE AND CREATININE CONTENT ON THE MUTAGENIC ACTIVITY OF MEAT EXTRACTS, BOUILLONS AND GRAVIES FROM DIFFERENT SOURCES

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Abstract—Thirteen commercial meat-flavour samples were analysed for creatine and creatinine content and tested for mutagenicity in the Ames Salmonella/microsome test. In most samples, more than 50% of the creatine had been converted to creatinine. Mutagenicity was related to the creatinine content: 150 μmol creatinine/g dry matter (gdm) gave upwards of 2500 revertants/gdm, concentrations of 15–40 μmol/gdm gave about 100 revertants/gdm and concentrations of 1–10 μmol/gdm gave only low or no significant mutagenicity. No relationship was apparent between coloration and mutagenicity. Beef steaks (initial weight *c.* 500 g) baked at oven temperatures between 115 and 245°C only showed significant mutagenicity—135 revertants/100 gE (initial raw weight)—in the crust when baked at the highest temperature (245°C). The gravies (meat-juice drip) collected during baking showed a linear increase in mutagenicity with baking temperatures up to 180°C (48–828 revertants/100 gE) and a very sharp increase in mutagenicity for the gravy collected from beef steak baked at 245°C (28,300 revertants/gdm or 19,800 revertants/100 gE). At this high temperature, the brown coloration and the proportion of creatinine to total creatine and creatinine were also dramatically increased, because this gravy dried up completely during the baking procedure.

INTRODUCTION

During the last decade several new highly mutagenic heterocyclic amines have been isolated from protein-rich foods and identified. Recent reviews show that some of these mutagenic compounds are found in the crust of fried, broiled or baked meat and fish as well as in different kinds of meat or beef extracts (Hargraves & Pariza, 1984; Miller, 1985; Sugimura, 1985). Moreover, some of the heterocyclic amines have shown a tumour-inducing capacity in mice and rats in long-term animal studies (Sugimura *et al.* 1986). The possible contribution of these compounds to the development of human cancer is now being extensively studied and evaluated all over the world.

Hitherto three different groups of mutagenic heterocyclic amines have been isolated. One group is made up of pyrolysis products from amino acids, especially tryptophan and glutamic acid (Sugimura *et al.* 1977; Yamamoto *et al.* 1978). Four different compounds in the second group, the 'IQ-type' compounds (IQ being 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline) have been identified in food (Felton *et al.* 1986; Takahashi *et al.* 1985). It has been proposed that these compounds are formed by Maillard reactions from monosaccharides, free amino acids and creatine or creatinine, since they consist of an imidazole group similar to creatinine and a pyridine or a pyrazine group. The two latter groups are typical Maillard reaction products (Jägerstad *et al.* 1983). When these low-molecular-weight precursors are heated together in model systems, all four compounds have been shown to be produced (Grivas *et al.* 1985 & 1986; Jägerstad *et al.* 1986; Muramatsu & Matsushima, 1985).

Felton *et al.* (1986) found that the IQ-type mutagens, mainly 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx) constituted 50% of the total mutagenicity in fried hamburgers, whereas 33% of the mutagenicity originated from a third group of pyridine compounds, of which trimethylimidazopyridine (TMIP) and phenylimidazopyridine (PhIP) have so far been identified. The precursors of these two compounds are not yet known, but it is interesting that the chemical structure of TMIP and PhIP, like that of the IQ-type mutagens, includes both imidazole and pyridine groups.

In Sweden, cooked meat products are often served with a gravy or broth prepared either from commercially prepared bouillon or from the meat juices produced during baking, roasting or frying. Commercial bouillon is also widely used in different kinds of soups. The meat flavour is derived from low-

Abbreviations: 4,8-DiMeIQx = 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline; DMSO = dimethylsulphoxide; gdm = grams dry matter; gE = grams initial raw weight; IQ = 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]-quinoline; MeIQx = 2-amino-3,8-dimethyl-3*H*-imidazo-[4,5-*f*]quinoxaline; PhIP = 2-amino-*N*-methyl-5-phenyl-imidazopyridine; TMIP = 2-amino-*N*,*N*,*N*-trimethyl-imidazopyridine.

molecular-weight compounds present in raw meat and the Maillard reaction is the principal reaction involved (MacLeod & Seyyedain-Ardebili, 1981).

Since creatine is one essential precursor for the formation of IQ in model systems, it is interesting to relate creatine content to mutagenicity in meat extracts. Creatine, monosaccharides and free amino acids occur naturally in meat of all species (Lawrie, 1979) but are present in higher concentrations in food-grade meat extracts (Sulser, 1978). Creatine is converted to creatinine during the heating of fish or meat (Hughes, 1960; Snider & Baldwin, 1981).

The first aim of the study reported here was to determine and compare the mutagenicity of samples derived from different sources and used commercially in bouillon, gravies and soups to develop the meat flavour. The samples were mainly commercial food-grade meat extracts, commercial bouillon and gravy samples based on food-grade meat extracts, commercial vegetable-based bouillon extracts, and gravies prepared after baking beef steaks at various oven temperatures. Secondly, the relationship between creatine/creatinine content and mutagenicity was evaluated and thirdly, brown coloration was determined as a measure of the Maillard reaction occurring during the processing of meat-flavour products. In addition mutagenic activity was measured in the crust of baked steaks.

MATERIALS AND METHODS

Materials

Commercial samples. Three commercial meat extracts of beef paste, of Argentinian origin and with a dry matter of 80%, were used (samples A, B and C). Sample D was a paste with a water content of 30%, declared as "natural pork flavour". One sample of canned beef consommé (E), dry matter 5%, was purchased locally and was lyophilized. Sample F was a powder declared as "meat flavour" and made synthetically from Maillard reaction products. All six samples were used directly for analysis. Cubes of bouillon and gravy samples, G, I, J, K and L, were purchased locally from two different stores and 12–18 cubes of each sample were dissolved in water, heated to cooking temperature for less than 10 min and chilled. The fat was separated by filtration through a filter paper and the samples were lyophilized before analysis. Two bouillon samples, H and M, in the form of freeze-dried powders were purchased locally at two different stores and were used directly for analysis. The meat bouillon samples and the gravy sample all contained meat extracts as an ingredient. The vegetable bouillon samples contained yeast extract. All bouillon samples also contained other ingredients such as salt, animal fat, hydrolysed vegetable protein, sugar, sugar colouring, vegetables, spices and 5′-nucleotides from inosine and guanosine, as well as antioxidants.

Baked beef and gravy—cooking procedures. Beef muscle (*Musculus longissimus dorsi*) was taken from a slaughterhouse about 2 days after slaughter. The muscles were taken from five different animals and were cut into 25 pieces of meat, each weighing around 400–500 g. The pieces were packed in vacuum packages and stored for 1–2 wk at +4°C before baking. The meat pieces were randomized before the baking, which was performed at five different temperatures. The possible influence of the composition and structure of different muscles and different ageing times could therefore be regarded as negligible.

Five pieces of meat were baked, one at a time, at each temperature, the crusts and gravies for each temperature being pooled. The meat pieces were placed on a gridiron and the meat juice was allowed to drip onto a stainless-steel plate. No fat, water or spices were added before or during the baking. Thermocouples were inserted, two in the oven and one in the centre of the pieces of meat and the temperatures were recorded. The meat pieces were conditioned to a temperature of 5–13°C before baking, and baking was stopped when the centre temperature had reached 72 ± 2°C. This required various times for various oven temperatures—about 90 min at 115°C, 75 min at 140°C, 59 min at 170°C, 50 min at 180°C and 47 min at 245°C. Each steak was weighed after baking and was then frozen to −20°C. While the steak was frozen, the crust, which was around 1 mm thick, was peeled off, weighed and lyophilized prior to analysis. The gravy or meat juice, which had dripped from the meat during baking, was collected, dissolved in tap-water, lyophilized and weighed.

Analytical procedures

Chemicals. Standard chemicals and reagents of analytical grade were purchased from Sigma Chemie GmbH (München, FRG), Kebo-Grave Labcenter (Stockholm) or Boehringer Mannheim (Mannheim, FRG). Synthetic MeIQx and 2-^{14}C-labelled MeIQx were gifts from Dr K. Olsson and Dr S. Grivas. The latter had a specific activity of 2.03 mCi/mmol and a radioactive purity of at least 96%. U-^{14}C-labelled histidine, obtained from the Radiochemical Centre (Amersham, Bucks, UK), had a specific activity of 336 mCi/mmole and a radioactive purity of 98%.

Meat samples. The water and fat content of the samples were analysed as described previously (Holtz *et al.* 1985). The pH of the different muscle samples ($n = 5$) was measured, also as described previously (Fabiansson & Laser Reuterswärd, 1985). Duplicates of the samples were homogenized in 0.6 M-perchloric acid, the supernatant was neutralized with $KHCO_3$ and creatine and creatinine were determined enzymatically in commercial extracts, crusts and gravies, as described by Wahlefeld *et al.* (1974). In this paper, creatin(in)e denotes the sum of creatine and creatinine. Raw-meat pieces ($n = 8$) were analysed for creatine and creatinine, free glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate, as described previously (Fabiansson & Laser Reuterswärd, 1985). Depth of colour was determined on duplicates of the perchloric acid extracts; it was measured spectrophotometrically at 375 nm and expressed as absorbance/g dry matter (Abs/gdm).

Extraction and recovery

Meat extracts, lyophilized samples of bouillons, lyophilized crusts and gravies from the baking experiments were all extracted for mutagenic activity assays by the method of Bjeldanes *et al.* (1982a). The

amounts of sample used were 15 g for the commercial samples, 20 g for crusts and 6–9 g for the corresponding gravies. Five samples were extracted at any one time and to control the reproduction of the extraction method, one sample of 15 g dehydrated Difco Bacto Nutrient Broth (beef extract 3 g, peptone 5 g; Difco Laboratories, Detroit, USA) was included each time.

Recovery in the extraction procedure was investigated by spiking 15 g Difco Beef Extract (dispersed in 40 ml 0.1 N-HCl) with 11.4 μg labelled MeIQx (240,000 dpm). The recovery was followed, from the homogenization until the extract was finally dissolved in dimethylsulphoxide (DMSO), by scintillation counting of the radioactivity using an LKB Wallac 1217 Rackbeta Liquid Scintillation Counter. Coloured samples were bleached with H_2O_2 before counting in accordance with the instructions given by the manufacturer of Instagel (Packard Instrument Co., Downers Grove, IL, USA). Counting efficiency was determined by external-standard ratios.

The recovery of added ^{14}C-labelled histidine was followed in an analogous way to check the possibility of histidine contamination of the samples.

Mutagenicity assay

Mutagenic activity was tested using *Salmonella typhimurium* strain TA98 as described by Ames *et al.* (1975) with activation by a rat-liver homogenate supernatant (S-9) prepared from rats treated with polychlorinated biphenyls (Aroclor 1254). To each plate, 0.5 ml S-9 mix containing 100 μl S-9 (20% S-9) was added. The appropriate amount of S-9 had previously been determined.

Prior to the Ames assays, samples were dissolved in 200 μl DMSO. They were then either used directly or after appropriate dilution with water. The bacteria, test sample and S-9 mix were incubated together for 20 min at 37°C before addition of top-agar. Each sample was analysed in duplicate at three levels—5, 10 and 20 μl/plate, and the assays were generally repeated at least once. As a positive control, synthetic MeIQx was used. The number of revertants/sample was calculated from the linear part of a dose-response curve as described by Bjeldanes *et al.* (1982a). A sample was considered mutagenic if it produced a dose-related increase in revertants compared to the zero-dose control and if the lowest increase was at least a doubling of the spontaneous reversion.

RESULTS

Control studies

The spiking of Difco Beef Extract with ^{14}C-labelled synthetic MeIQx showed an average recovery ($\pm$SEM) of 83 $\pm$ 13% ($n = 4$) after purification on an SM-2 Bio Bed column. In addition the homogenization procedure gave an average loss of 10%. Spiking with ^{14}C-labelled histidine showed no radioactivity in the fraction usually used for the mutagenicity assay, so it seems unlikely that the food samples assayed were contaminated with any histidine.

The mean value of seven separate analyses of Difco Beef Extract was 2484 revertants/gdm (SEM 175) The corresponding result for the positive control, synthetic MeIQx, was 50,000 revertants/μg (SEM 2494), which is close to our previous results (Grivas & Jägerstad, 1984).

Commercial meat extracts, bouillons and gravies

Table 1 shows the creatinine and creatin(in)e contents, mutagenicity and colour for all the commercial samples analysed. The creatin(in)e levels varied between zero and about 850 μmol/gdm. The mutagenicity of the meat extracts (A, B and C) varied between 2500 and 10,000 revertants/gdm. That of the two meat flavour products (D and F), the soup (E) and the bouillon and gravy samples (G–M) ranged from insignificant levels up to 300 revertants/gdm. Of the 13 commercial samples totally analysed, five showed no significant mutagenic activity. Difco Beef Extract had a mutagenicity value of 2500 revertants/gdm.

The colour value was highest in the synthetic meat flavour sample, with an absorbance of 76 Abs/gdm, whereas the meat extracts had values of about 50 Abs/gdm, the meat bouillon, soup and gravy samples of 10–35 Abs/gdm and the vegetable bouillons of only about 7 Abs/gdm.

The crust and gravy of baked beef steak

The raw meat (water 74%, fat 3%) used in the baking experiment had a pH of 5.5–5.6. The levels (in μmol/g wet tissue) of glucose and its metabolites were found to be glucose 8.3 $\pm$ 1.1, glucose-6-phosphate 10.8 $\pm$ 1.1, glucose-1-phosphate 0.37 $\pm$ 0.10 and fructose-6-phosphate 2.2 $\pm$ 0.29. The pH and metabolite values were the same as were found in normal meat (Fabiansson *et al.* 1984; Fabiansson & Laser Reuterswärd, 1985). The creatine and creatinine levels of the raw meat were found to be 34 and 1.9 μmol/g wet tissue, respectively, corresponding to a total creatine content of 0.45% of wet tissue. This value is in accordance with results of earlier analyses of the same type of muscle (Olsman & Slump, 1981).

Processing data for the beef steaks cooked at the five different oven temperatures are shown in Table 2. The amounts of crust were found to account for 8–11% of the steak when calculated as wet crust/100 g baked steak, for 34–38% when calculated as dry matter of crust/dry matter of baked steak and for about 2% (Table 2) when calculated as dry matter of crust/100 gE (initial raw weight). The total amounts of crust obtained were thus the same for all oven temperatures, while the amounts of gravy varied, between 0.39 and 0.80 gdm/100 gE. The temperature profiles in the oven and in the centre of the meat during baking are shown in Fig. 1.

Table 3 shows the amounts of creatine and creatinine, the mutagenicity and the colour values for baked beef steaks. In the crusts, the creatine level decreased and the creatinine level increased with temperature. However, creatin(in)e decreased with temperature and a loss was thus indicated. In the gravies the same pattern was observed, with creatine decreasing, creatinine increasing and creatin(in)e decreasing. The creatine and creatinine concentrations were considerably higher in the gravies than in the crusts.

Table 1. Creatinine and creatin(in)e levels, mutagenicity and colour of commercial samples of meat extracts, bouillons and gravy

	Sample	Animal origin	Form	Manufacturer	Creatinine (μmol/gdm)	Creatin(in)e* (μmol/gdm)	Creatine/ creatin(in)e (%)	Mutagenicity† (revertants/gdm)	Coloration (absorbance/ gdm)
A	Meat extract	Beef	Paste	(1)	301	593	51	9800	41.0
B	Meat extract	Beef	Paste	(2)	465	849	55	6900	54.7
C	Meat extract	Beef	Paste	(3)	149	719	21	2500	57.4
D	Meat flavour, natural	Pork	Paste	(4)	5.1	5.1	100	NS	62.3
E	Consommé	Beef	Soup	(5)	24.8	43.4	57	122	22.1
F	Meat flavour, synthetic	—	Powder	(6)	0	0	—	288	76.0
G	Meat bouillon	Beef	Cube	(7)	37.2	44.3	84	170	18.8‡
H	Meat bouillon	—	Powder	(8)	14.2	27.5	52	106	10.6‡
I	Meat bouillon	—	Cube	(7)	11.1	20.0	55	NS	10.9‡
J	Meat bouillon	Beef	Cube	(7)	5.5	7.3	75	NS	34.7‡
K	Meat gravy	Beef	Cube	(7)	3.6	8.5	42	NS	12.0‡
L	Vegetable bouillon	—	Cube	(7)	2.5	3.7	68	NS	7.5‡
M	Vegetable bouillon	—	Powder	(8)	1.8	2.0	90	42	6.4‡
N	Difco Beef extract	Beef	Powder	(9)	—	—	—	2500	—

gdm = Grams dry matter NS = Not significant

*Sum of creatine and creatinine.
†To *Salmonella typhimurium* in the presence of S-9 mix.
‡Sugar colouring added.

It was possible to determine the relative amounts of creatine converted to creatinine during heating. The quotient creatinine/creatin(in)e in raw muscle was very low (only 5%) but during heating the proportion of creatinine in the crust increased with temperature, the creatinine/creatin(in)e ratio being 39% at 245°C. The proportion was higher in the corresponding gravies, rising to 85% at 245°C.

No significant mutagenicity was recorded in any crusts except those produced at 245°C, which showed a value of about 135 revertants/100 gE. However, mutagenicity was found in all the gravy samples. It did not exceed 1000 revertants/100 gE when the beef steaks were baked at or below 180°C, but in the 245°C gravy it was very high—about 20,000 revertants/100 gE or some 25 times higher than at 180°C. Brown coloration increased with temperature both in crusts and gravies, the colour value being higher in the gravies than the crusts. A marked colour effect was obtained in the 245°C gravy.

DISCUSSION

This investigation shows a wide variation in the Ames test mutagenicity of commercial meat extract, bouillon and gravy samples. This is in accordance with published values of 3000–5500 revertants/g for food-grade meat extracts, corresponding to our meat extracts (Commoner *et al.* 1978b; Hargraves & Pariza, 1983). Beef broth and beef bouillon, corresponding to our bouillon and gravy samples, were reported in a review by Hargraves & Pariza (1984) to have low values of 2–5 revertants/gdm. Difco Beef Extract showed values between 1111 and 25,000 revertants/gdm (Hargraves & Pariza, 1984) while our sample induced about 2500 revertants/gdm. Münzner (1981) also found wide variations in the mutagenicity of samples of commercial bouillon cubes used for soups as well as of meat extracts for bacterial use.

The variation in the mutagenicity of meat extracts, beef stock and bouillons depends on several factors, including the processing conditions, such as time, drying temperature and water content (Aeschbacher *et al.* 1985; Commoner *et al.* 1978a; Dolora *et al.* 1979; Münzner, 1981) and the amounts of precursors.

According to Sulser (1978), a commercial food-grade meat extract should meet certain criteria, particularly a maximum water content of 20%, maximum fat content of 2% and a total creatine content of 5–7%. The meat extracts A, B and C and had a water content of 20% and the total creatine levels were found to be 5.9, 8.3 and 6.5%, respectively. In some countries, criteria for the total creatine content of bouillon and gravy samples exist but for meat-flavour samples there are no such criteria.

No data seem to be available on the influence of the chemical composition of meat-flavour products on their mutagenicity. In this study, the meat extracts A, B and C showed no direct relationship between creatinine, creatin(in)e and mutagenicity. However, it is evident that a low creatin(in)e level, as in the meat bouillon samples, was associated with mutagenicity values that were low or not significant. Thus, while a concentration of more than 150 μmol creatinine/gdm induced more than 2500 revertants/gdm, concentrations of 14–37 μmol/gdm gave values of about

Table 2. Processing data for the baking of beef steaks at various oven temperatures

Temperature (°C)		Baking time (min)	Steak weight*		Total yield:	
Oven	Centre of meat		Initial weight (g)	Weight loss (%)	Of crust (gdm/100 gE)	Of gravy (gdm/100 gE)
115	70.5	90	466 ± 34	30 ± 2.6	2.7	0.63
140	71.0	75	458 ± 43	25 ± 3.6	1.9	0.39
170	71.0	59	442 ± 46	22 ± 8.6	2.2	0.47
180	74.5	50	470 ± 32	33 ± 2.4	2.2	0.80
245	76.5	47	482 ± 30	30 ± 1.4	2.0	0.67

gdm = Grams dry matter gE = Grams initial wet weight

*Values are means ± SEM for five steaks.

100 revertants/gdm and 1–*c*.10 μmol/gdm creatinine was associated with low or not significant levels of mutagenicity. It is interesting to note that the other ingredients of the bouillon samples included in this study (such as vegetables, sugar colouring and hydrolysed vegetable protein) do not seem to contribute to any significant mutagenicity in the Ames test. This is in accordance with the results of Scheutwinkel-Reich & von der Hude (1985) and Aeschbacher (1986), who reported that sugar colorant or caramelized products, for example, showed no mutagenicity, or only low values.

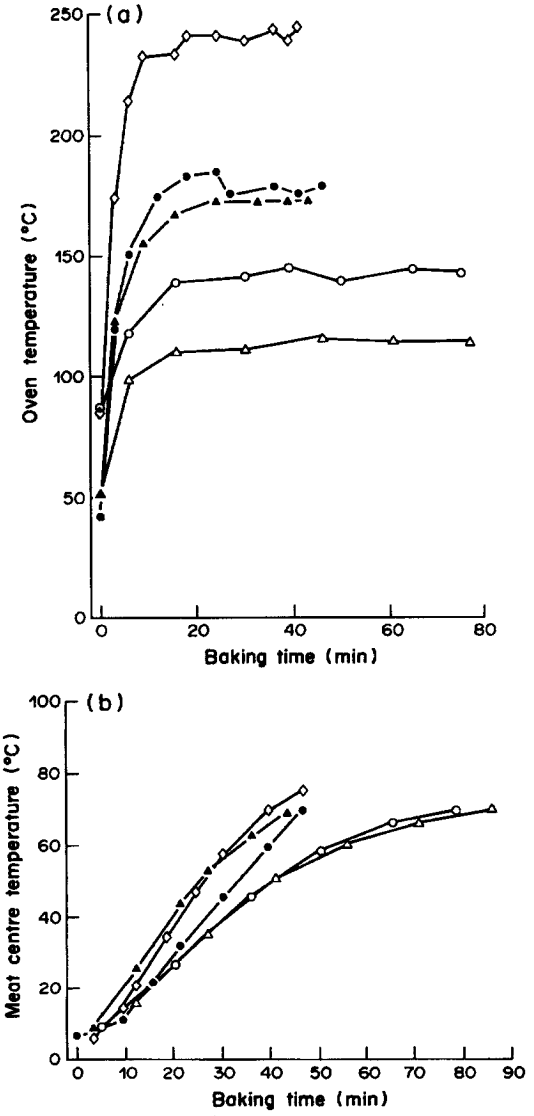

Fig. 1. Temperatures recorded (a) in the oven and (b) in the centre of the meat during the baking of beef steaks at nominal oven temperatures of 115 (△), 140 (○), 170 (▲), 180 (●) and 245 (◇) °C.

Our results indicate, therefore, that creatine and/or creatinine content is linked with the development of mutagenicity in meat-extract bouillon and gravy samples. Creatine only occurs in samples of animal origin (Sulser, 1978). Phosphocreatine, found in most vertebrates, is involved in the energy metabolism of muscle (Lehninger, 1970), but in meat the phosphorus group splits off from creatine within 2 days after slaughter (Fabiansson & Laser Reuterswärd, 1985). In a model system, IQ was produced when creatine phosphate was added before boiling (Taylor *et al.* 1985).

We have no explanation for the small amounts of creatine and creatinine found in our two vegetable bouillon samples (L and M). Sample M also showed a low level of mutagenicity.

The meat-flavour sample F contained no creatin(in)e, but showed some mutagenicity (300 revertants/gdm). This flavouring product was prepared by cooking amino acids with yeast extract containing sugar, the classic reactants of the Maillard reaction. There are several reports of the mutagenicity of such model systems in TA98 in the presence of S-9 activation (Matsushima, 1982; Shibamoto *et al.* 1981; Spingarn & Garvie, 1979; Yoshida & Okamoto, 1980),

Commercial food-grade meat extract is never used for direct consumption, but forms the basis of commercial bouillon cubes and powders. Bouillon samples can thus be considered as products in which meat extract is diluted and hence the mutagenic activity, derived from meat extract, is also diluted. In our analyses of the bouillon samples, therefore, any mutagenic activity derived from the meat extract was probably undetectable by the Ames test.

Coloration did not correlate with mutagenicity in the 13 commercial samples analysed. Sample F, with significant but medium mutagenicity, showed the highest colour value, which resulted from an effective Maillard reaction with concentrated amounts of sugar and amino acids. Sugar colouring had been added to the bouillon samples and therefore no relationship with the Maillard reaction was involved in these cases. In the three meat extracts, the highest colour value was associated with the lowest mutagenicity. When relating coloration to mutagenicity,

Table 3. Creatine and creatinine levels, mutagenicity and colour absorbance of the crust and gravy from beef steaks baked at various oven temperatures

	Oven temp. (°C)	Concns in steaks: Creatine (μmol/gdm)	Creatinine (μmol/gdm)	Creatin(in)e* (μmol/gdm)	Creatinine/ creatin(in)e (%)	Mutagenicity* (no. of revertants): No./gdm	No./100 gE	Coloration (absorbance/ gdm)
Meat	Raw	138	7.7	146	5	—	—	—
Crust	115	111	11	122	9	NS	NS	0.71
	140	109	11	120	9	NS	NS	0.78
	170	103	12	115	10	NS	NS	0.65
	180	83	17	100	17	NS	NS	1.3
	245	68	29	97	39	68	135	2.3
Gravy	115	554	103	657	16	76	48	5.5
	140	536	194	730	27	1146	435	23
	170	314	348	662	53	1523	716	56
	180	—	—	—	—	1035	828	28
	245	67	375	442	85	28,300	19,800	172

gdm = Grams dry matter NS = Not significant

*Sum of creatine and creatinine

†To *Salmonella typhimurium* in the presence of S-9 mix.

only samples produced under controlled or known conditions can be evaluated. Commoner *et al.* (1978a) showed a relationship between optical density and mutagenicity in beef stock cooked for different lengths of time, and a relationship between mutagenicity and coloration in pan-fried patties and in meat loaves baked under various oven conditions has also been shown (Holtz *et al.* 1985; Jägerstad *et al.* 1983).

In the crusts of beef steaks significant but low mutagenicity was produced only after baking at the highest temperature (245°C). This agrees with the data of Bjeldanes *et al.* (1982b), who found 150 revertants/100 gE in tests on a round beef steak baked at 176°C for 60 min, while after 90 min at 176°C mutagenicity was increased considerably, to 3350 revertants/100 gE. In one unpublished experiment, we found 4300 revertants/100 gE were induced by the crust of a beef steak initially weighing 1800 g and baked at 200°C for 130 min. Baking minced meat with a fat content of 14% at an oven temperature of 200°C and final surface temperature/time combinations of 143°C/50 min, 150°C/59 min and 170°C/97 min gave mutagenicity values of 5000, 8000 and 8000 revertants/100 gE, respectively (Holtz *et al.* 1985). The fact that these values are higher than the data obtained in the present study can probably be ascribed to the higher fat content of the samples used by Holtz *et al.* Fat has been proposed as an efficient vehicle for heat transfer, producing high temperatures in the crust more rapidly (Bjeldanes *et al.* 1983; Holtz *et al.* 1985). Felton & Hatch (1986) pointed out that pan-frying and broiling produce much higher levels of mutagenicity than other cooking procedures, including baking.

Of the gravies collected during baking, that collected from beef steaks baked at 245°C showed a very sharp rise in mutagenicity. Brown coloration and the proportion of creatinine/creatin(in)e were also dramatically increased at this high temperature. This was probably because this was the only one of the five gravies that totally dried out during baking. A low water content is considered to favour Maillard reactions involving dehydration (Mauron, 1981). No baking experiments in which the gravy has been subjected to mutagenicity assays seem to have been published previously. In one unpublished experiment in which we baked beef steak (initial weight 1200 g) at 200°C for 90 min, the collected gravy, which had also dried out, showed a mutagenicity value of 34,000 revertants/gdm. The total amount of gravy produced was 12.7 g, with a comparable revertant-induction level to that of gravy baked at 245°C in the experiment reported here.

In a study on pan-fried pork (5% fat), Övervik *et al.* (1986) found similar levels of mutagenicity in the gravy and crust. Pan-frying at 200°C for 10 min with and without addition of frying fat yielded products giving a total of 25,000 and 17,000 revertants/100 gE, respectively (the sum of the mutagenic activities in the crust and gravy). At 250°C the corresponding figures were 52,000 and 26,000 revertants/100 gE. Thus gravy produced without added fat induced 8500–13,000 revertants/100 gE which is less than the level of mutagenicity demonstrated for the gravy produced at 245°C in our experiment.

In the study reported here, the concentrations of creatine and creatinine were considerably higher in gravies than in crusts, indicating an increased level of the low-molecular-weight creatine in meat juice that had dripped during baking. The concentration of creatinine in the gravy at 140–245°C was similar to that in the commercial meat extracts A, B and C (Table 1). However, creatin(in)e was generally somewhat higher in the meat extracts, probably because of differences in the processing of the samples. The gravy was produced in about 1 hr, while commercial meat extract is usually cooked for several hours at lower temperatures before evaporation (Sulser, 1978).

During baking a large part of the creatine was converted to creatinine and both compounds were lost during heating. Similarly, Hughes (1960) found that in heated herring 25% of the creatine was converted and total loss of creatine and creatinine occurred. Snider & Baldwin (1981) found that about 13% of creatine was converted when beef was ordinarily cooked and the whole steak was analysed. Since crust reaches a higher temperature than the inside of the steak, the lower conversion found by Snider & Baldwin (1981), compared with our results, seems reasonable.

Natural meat flavour can be obtained from bouillon, soup or gravy. From the numbers of revertants obtained in our study, samples E, F, G and H would give about 115–300 revertants/100 g liquid when used in the concentrations recommended by the manufacturer. The baking experiment indicates that gravies, as used, would give after baking at 140–180°C about 400–800 revertants/100 gE but after baking at 245°C about 20,000 revertants/100 gE, this amount of gravy being roughly equivalent to one portion. Dried gravy thus shows the highest mutagenicity in the Ames test.

It is well known that meat flavour is derived from low-molecular-weight water-soluble substances and that the Maillard reaction is the principal reaction (MacLeod & Seyyedain-Ardebili, 1981; Sulser, 1978). However, the role of creatine in the development of meat flavour is controversial. Sulser (1978) reviews some papers indicating that creatine has a positive effect on meat flavour but results by Snider & Baldwin (1981) show that this compound is not important for the flavour intensity of cooked beef. A lot of substitutes for meat flavour on the market are not based on creatine. MacLeod & Seyyedain-Ardebili (1981) pointed out that it is still very difficult to produce meat flavours in the absence of some of the natural product or a meat extract.

In conclusion, the study reported here clearly demonstrates the impact of creatin(in)e content on the mutagenicity of samples used to develop meat flavour. For the commercial meat extract and gravy samples a total concentration of creatine plus creatinine of at least 700 μmol/gdm is required in order to obtain more than 10^3 revertants/gdm. The gravies collected during baking showed a linear increase in mutagenicity with temperatures up to 180°C, followed by a very sharp increase in mutagenicity for the gravy collected from beef steak baked at 245°C. Brown coloration and the proportion of creatinine/creatin(in)e were also dramatically increased at this high temperature because this gravy dried up during the baking process. Thus, both for the model systems known to produce IQ-type mutagens and for meat extract, bouillon and gravy samples, mutagenicity is dependent on the presence of creatine or creatinine.

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MUTAGENICITY OF PAN-FRIED BOVINE TISSUES IN RELATION TO THEIR CONTENT OF CREATINE, CREATININE, MONOSACCHARIDES AND FREE AMINO ACIDS

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Abstract—The mutagenicity of pan-fried patties of five bovine tissues (meat, heart, tongue, liver and kidney) containing various concentrations of creatine, monosaccharides and free amino acids were studied. Two experiments were performed, one on single tissues fried at 150, 175 or 200°C for 3 min and the other on mixtures of meat and one of the other four tissues in various proportions, fried at 200°C for 3 min. For both experiments, a double-sided Teflon-coated plate was used. Frying at 150°C induced mutagenicity to *Salmonella typhimurium* strain TA98 only in the heart sample—6000 revertants/100 gE (grams initial raw weight). Meat, heart and tongue fried at 175 or 200°C showed mutagenicity values between 6000 and 19,600 revertants/100 gE. A linear relationship between mutagenicity and temperature was obtained for each of the three muscles and creatine was converted to creatinine with increasing temperature. Liver or kidney samples fried alone showed insignificant mutagenicity at all three temperatures. The creatine plus creatinine levels of raw meat, heart and tongue samples were between 19 and 33 μmol/g wet tissue. Liver and kidney both showed very low amounts of creatine plus creatinine (about 2 μmol/g wet tissue) in the raw tissue, while free amino acids were high. Glucose levels were high in liver but low in kidney samples. In meat/heart and meat/tongue mixtures the mutagenicity varied between 10,800 and 17,300 revertants/100 gE. The meat/liver and meat/kidney mixtures showed linear relationships between mutagenicity and the proportions of the mixture. The values for the slopes and intercepts of the two lines were almost equal. Among the three groups of precursors (creatine plus creatinine, monosaccharides and free amino acids) the creatine plus creatinine in raw tissue seems to be the most important for producing mutagenicity. However, in crusts, the creatinine concentration was the variable with which most of the mutagenicity was associated.

INTRODUCTION

In a model system, creatine and/or creatinine together with monosaccharides and free amino acids act as precursors of mutagenic IQ compounds, IQ being 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (Laser Reuterswärd *et al.* 1987). The IQ-type mutagens belong to a class of crust mutagens that are produced during the cooking of various meat products. Moreover, samples of fried beef and meat flavour products such as meat extract, bouillon and gravies show levels of mutagenicity that vary with the concentrations of glucose or creatine and creatinine (Jägerstad *et al.* 1983a; Laser Reuterswärd *et al.* 1987).

The study reported here extends this work to include the frying of various bovine tissues such as meat, heart, tongue, liver and kidney, selected not only because of their wide variation in the concentrations of creatine in the raw tissue (Olsman & Slump, 1981) but also because of the varying concentrations of monosaccharides and free amino acids. The main purpose of this study was to examine how the concentrations of these precursors (creatine, creatinine, monosaccharides and free amino acids) affect the levels of mutagenicity produced by moderate pan-frying of patties of the various bovine tissues at between 150 and 200°C for several minutes. The mutagenicity, as measured with the Ames Salmonella/microsome test, was also examined in relation to the development of a brown coloration (the Maillard reaction).

Abbreviations: DMSO = dimethylsulphoxide; gdm = grams dry matter; gE = grams initial raw weight; MeIQx = 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoline.

MATERIALS AND METHODS

Bovine tissue experiment. Bovine tissues, namely meat (*Musculus longissimus dorsi*), heart, tongue, liver and kidney, were taken from a slaughterhouse, within 1 wk of slaughter. The samples, freed from visible fat, were ground and formed into approximately 10-mm thick patties, weighing 50 g each. The patties consisted of either of a single tissue or of a mixture of meat with either heart, tongue, liver or kidney, in each case in proportions of 90/10, 75/25 and 60/40 (w/w). One patty at a time was pan-fried on a double-sided Teflon-coated plate for 3 min. The single-tissue samples were fried at 150, 175 or 200°C

Table 1. Design of frying experiments and processing data for ground bovine tissues fried as 50-g patties on a double-sided Teflon-coated plate

Patty composition		Frying conditions		Weight loss (%)	Thickness (mm) after frying		Total yield of crust (gdm/100 gE)
Tissue	Mixture (%)	Temp. (°C)	Time (min)		Patty	Crust	
				Experiment 1			
Meat	100	150	3	47	6.5	1.0	6.8
		175	3	50	6.5	1.0	5.9
		200	3	52	6.5	1.0	7.0
Heart*	100	150	3	62	5.8	—	19.4
		175	3	68	4.5	—	19.4
		200	3	71	3.8	—	19.4
Tongue*	100	150	3	62	5.6	—	23.5
		175	3	66	5.3	—	24.4
		200	3	69	4.3	—	25.1
Liver*	100	150	6	64	3.0	—	24.9
		175	3	53	5.5	—	26.9
		200	3	59	4.5	—	25.0
Kidney*	100	150	3	63	3.8	—	19.2
		175	3	69	3.1	—	20
		200	3	68	3.6	—	20
				Experiment 2			
Meat/heart	90/10	200	3	48	7.0	1.0	5.2
	75/25	200	3	52	6.7	1.0	7.4
	60/40	200	3	54	6.5	1.0	4.9
Meat/tongue	90/10	200	3	47	7.0	1.0	7.5
	75/25	200	3	51	6.7	1.0	6.8
	60/40	200	3	52	6.3	1.0	7.2
Meat/liver	90/10	200	3	45	7.2	1.0	10.4
	75/25	200	3	47	7.2	1.0	8.3
	60/40	200	3	48	6.9	2.0	10.1
Meat/kidney	90/10	200	3	46	7.1	1.0	5.5
	75/25	200	3	47	7.2	1.0	6.7
	60/40	200	3	49	6.2	1.0	6.5

gdm = grams dry matter gE = grams initial raw weight
*Crust was not peeled off after frying.

and the mixtures at 200°C. For each temperature or mixture, five samples were fried and pooled. No fat was added before or during the frying, nor were any spices or other food ingredients added to the patties. After frying, the patties were weighed and frozen. For some samples the crust was peeled off (a thickness of 1–2 mm from each side) and weighed. For other samples, the whole patty was used for analysis and regarded as crust. The samples were minced, lyophilized and stored at −20°C until analysis. The experimental designs are outlined in Table 1.

Chemical analyses. All analyses were performed in duplicate on lyophilized samples using analytical-grade chemicals and reagents. The water, fat, protein and ash of raw samples were determined according to standard methods, as previously described (Holtz *et al.* 1985). Creatine, creatinine and coloration were determined on raw and cooked samples, as described previously (Laser Reuterswärd *et al.* 1987). Raw bovine tissues were analysed for glucose, glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate, as already described (Fabiansson & Laser Reuterswärd, 1985). Free amino acids, dipeptides (anserine and carnosine) and other amino compounds were determined in lyophilized powders of raw bovine tissues using ion-exchange chromatography, which was performed at the Central Amino Acid Analysis Laboratory, Biomedical Centre, Uppsala.

Extraction, recovery and analysis of mutagenicity. Lyophilized powders of the various crusts were extracted for estimation of mutagenic activity using the method of Bjeldanes *et al.* (1982a). After evaporation, the acetone phase was dissolved in 200 μl dimethylsulphoxide (DMSO). Samples of 10–15 g were used. The recovery achieved in the extractions was determined by spiking crumb and crust with 2-^{14}C-labelled 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx), as described previously (Laser Reuterswärd *et al.* 1987). Mutagenic activity was determined according to Ames *et al.* (1975) using the *Salmonella typhimurium* strain TA98 as the test organism, with and without activation by rat liver (S-9) homogenate, as described previously (Laser Reuterswärd *et al.* 1987). A substance was considered mutagenic if it produced a dose-related increase in revertants compared to the zero-dose control and if the lowest increase was at least a doubling of the spontaneous reversion.

Statistics. Stepwise multiple regression analyses were performed to explain variations in mutagenicity and colour and their dependence on tissue composition and other variables monitored in the crust (Draper & Smith, 1981).

RESULTS

Composition of raw bovine tissues

Table 2 shows the chemical composition of the raw bovine tissues. The water content varied between 72 and 81% and the fat content was 1–3% for all samples except tongue, which contained about 12%. Protein constituted 15–21% and the ash was about 1%. Liver contained an analytical residue of 6%,

Table 2. Chemical composition of raw bovine tissues

Component*	Concentration in				
	Meat	Heart	Tongue	Liver	Kidney
Water (%)	74.3	80.7	72.0	71.5	80.6
Fat (%)	3.3	1.5	11.7	2.2	1.4
Protein (%)	21.0	16.3	14.6	19.0	15.5
Ash (%)	0.8	1.1	0.9	1.4	1.1
Analytical residue (%)	0.7	0.4	0.8	5.9	1.4
Monosaccharides (μmol/g)	12	2.0	9.3	183	0.7
Glucose	8.2	1.6	7.4	183	0.45
Glucose-6-phosphate	3.0	0.25	1.4	0.30	0.27
Glucose-1-phosphate	0.15	0.07	0	0	0
Fructose-6-phosphate	0.75	0.07	0.47	0	0
Creatin(in)e† (μmol/g)	33	25	19	2.2	2.3
Creatine	31	23	17	1.7	1.8
Creatinine	2.0	1.9	2.0	0.50	0.47
Dipeptides (μmol/g)	23	0.4	2.2	0.8	0
Carnosine	21	0.4	1.5	0.8	0
Anserine	2.0	0	0.7	0	0
Total free amino acids (μmol/g)	30	37	46	101	71

*Concentrations of all components are calculated on the wet tissue.
†Creatin(in)e = creatine plus creatinine.

which was probably due to the glycogen content. Thus all the five bovine tissues had a relatively similar content of water, fat, protein and ash-generating material. However, the concentrations of the low-molecular-weight compounds varied considerably between the raw bovine tissues. The total concentration of monosaccharides (glucose, glucose-6-phosphate, glucose-1-phosphate and fructose-1-phosphate) was highest in liver (183 μmol/g wet tissue) followed by meat (12 μmol/g) and tongue (9 μmol/g) and lowest in the heart (2 μmol/g) and kidney (0.7 μmol/g). The levels of glucose and phosphate-bound monosaccharides in the meat sample were the same as those normally found in meat (Fabiansson & Laser Reuterswärd, 1985; Laser Reuterswärd *et al.* 1987). Creatine plus creatinine levels in meat, heart and tongue samples were 33, 25 and 19 μmol/g wet tissue, respectively, while liver and kidney contained only about 2 μmol/g wet tissue of creatine and less than 0.5 μmol creatinine. In the three muscles the creatine content was considerably higher than the creatinine content. The creatine levels and proportions in the different tissues were in agreement with those found by Olsman & Slump (1981). Meat was found to be the richest source of dipeptides, in total 23 μmol/g wet tissue, while almost negligible amounts were

Table 3. Composition of free amino acids and other free amino compounds in bovine tissues

Amino compound	Concentration (μmol/g wet tissue) in:					
	Meat	Heart	Tongue	Liver	Kidney	Reactivity*
Taurine	1.3	3.6	24	7.3	5.0	—
Phosphoethanolamine	—	0.38	—	—	0.30	—
Urea	—	4.6	1.0	—	13.2	—
Aspartic acid	0.09	0.38	0.28	3.0	2.9	(13)
Threonine	0.87	0.29	0.35	2.8	1.5	(1)
Serine	1.4	0.70	0.63	4.4	2.2	(5)
Aspargine	0.44	0.17	0.27	2.1	0.43	(11)
Glutamic acid	1.05	1.8	0.70	19	11	(20)
Glutamine	6.1	14	6.4	0.45	0.57	(18)
Proline	0.47	0.34	0.30	3.3	2.0	(19)
Glycine	1.5	0.80	1.02	15.3	9.6	(2)
Alanine	5.1	6.0	5.2	9.3	4.6	(4)
Citrulline	—	—	—	—	0.22	—
Valine	1.4	0.39	0.52	3.7	1.7	(9)
Cystine	—	—	—	—	0.50	(21)
Methionine	0.70	0.15	0.20	1.5	0.60	—
Isoleucine	0.82	0.17	0.29	2.0	0.86	—
Leucine	1.6	0.48	0.67	5.0	2.1	(6)
Tyrosine	0.30	0.10	0.07	1.5	0.65	(12)
β-Alanine	—	—	0.13	1.1	0.75	—
Phenylalanine	0.70	0.20	0.25	2.1	0.83	(14)
γ-Aminobutyric acid	—	—	—	—	—	—
Ethanolamine	—	—	—	—	1.6	—
Ammonia	3.4	1.8	3.0	7.1	2.8	—
Ornithine	0.19	0.18	0.33	3.3	1.4	—
Lysine	1.0	0.36	0.38	4.6	2.1	(3)
1-Methylhistidine	—	—	—	—	0.22	—
Histidine	0.41	0.25	0.23	1.7	0.76	(7)
Arginine	1.0	0.31	0.12	0.14	0.28	(8)
Total...	30	37	46	101	71	

*Reactivity order for the mutagenicity of free amino acids in a model system of creatine, glucose and amino acid, refluxed at 128°C (Jägerstad *et al.* 1983a).

Table 4. Frying experiment 1: amounts of creatine and creatinine, mutagenicity data and colour values for ground bovine tisues fried for 3 min at different temperatures on a double-sided Teflon-coated plate

Sample origin	Temperature (°C)	Creatine (μmol/gdm)	Creatinine (μmol/gdm)	Creatin(in)e* (μmol/gdm)	Creatinine/ Creatin(in)e (%)	Mutagenicity (no. of revertants)		Coloration (Abs/gdm)
						No./gdm	No./100 gE	
Meat	Raw	120	7.9	128	6	—	—	—
	150	72.9	36.6	110	33	NS	NS	3.0
	175	53.3	47.8	101	47	1044	6160	4.0
	200	45.1	70.9	116	61	1529	10703	5.8
Heart†	Raw	121	9.8	131	8	—	—	—
	150	54.6	28.5	83	34	302	5859	1.2
	175	49.4	40.7	90	45	444	8614	1.2
	200	47.5	45.0	92	49	800	15520	1.1
Tongue†	Raw	62.1	7.0	69	10	—	—	—
	150	43.7	20.7	64	32	NS	NS	1.8
	175	26.1	28.3	54	52	507	12371	2.2
	200	15.8	26.6	42	63	781	19603	2.6
Liver†	Raw	5.9	1.8	7.7	23	—	—	—
	150	1.8	2.4	4.2	57	NS	NS	6.6
	175	2.3	2.1	4.4	48	NS	NS	4.4
	200	2.1	2.4	4.5	53	NS	NS	5.6
Kidney†	Raw	9.4	2.4	11.8	20	—	—	—
	150	4.5	3.1	7.6	41	NS	NS	1.0
	175	4.0	4.5	8.5	53	NS	NS	1.1
	200	3.7	4.4	8.1	54	NS	NS	0.9

gdm = grams dry matter gE = grams initial raw weight NS = not significant

*Creatin(in)e = creatine plus creatinine.

†Crust was not peeled off after frying.

found in the other four tissues. The carnosine content in meat was found to be ten times higher than the anserine content, again in accordance with the results of Olsman & Slump (1981), who also reported low concentrations of dipeptides in the liver and heart.

Table 3 shows the concentrations of free amino acids and other free amino compounds in the raw tissues. The total concentrations of free amino acids in liver and kidney (101 and 71 μmol/g wet tissue) were about twice the concentrations in the three muscular tissues, meat, heart and tongue (30–46 μmol/g).

Fried patties of individual bovine tissues

Table 1 shows the design of frying experiment 1 which was performed at different temperatures. In addition, data on weight loss, patty and crust thickness and yield of crust are shown.

The weight losses for the meat samples were 40–50%, and for the other tissues 50–70%. The patty thickness after frying ranged from 3 to 7 mm. The crusts on the meat patties were peeled off and thus the total yield of crust was lower than for the other bovine patties, about 7 g dry matter (gdm)/100 gE (initial raw weight) compared with 20–25 gdm/gE.

Table 4 shows the amounts of creatine and creatinine, the mutagenicity and the colour values after frying. For meat crusts, as well as for all the other four bovine-tissue patties, the amount of creatine decreased while that of creatinine increased with higher frying temperature. For example, for meat and heart, creatine decreased from about 120 μmol/gdm in raw tissue to about 45 μmol/gdm after frying at 200°C. The corresponding creatinine values increased from about 10 to more than 45 μmol/gdm. Creatine was thus converted to creatinine with increasing temperature. However, the values for creatine plus creatinine (creatin(in)e) in raw samples were higher than in the heated samples, indicating losses during pan-frying. The values for different temperatures were inconsistent, but showed a tendency for increasing loss with increasing temperature especially in the tongue samples. For a total of nine samples of meat, heart and tongue, a linear relationship was obtained between the proportion of creatinine/creatin(in)e and temperature (Fig. 1).

During the extraction procedure for determining mutagenicity, an average recovery of 70–75% ($n = 3$) after purification on an SM-2 Bio Bed column was obtained for crust and crumb. In addition the homogenization procedure gave an average loss of 10%.

All the mutagenicity data shown in Table 4 were calculated from dose-response curves. At 150°C, patties prepared from meat or tongue did not show any significant mutagenicity. For heart samples cooked at this temperature, however, a value of about 6000 revertants/100 gE was recorded. Mutagenicity was

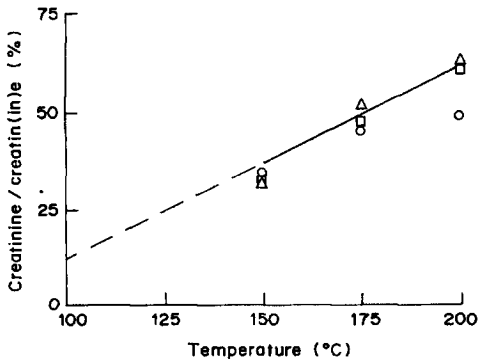

Fig. 1. Linear relationship between the proportion of creatinine/creatin(in)e (creatine plus creatinine) and the frying temperature for samples of meat □, heart ○ and tongue △. The equation was defined as $y = 0.005x - 0.382$, and the correlation coefficient as 0.92 ($n = 9$).

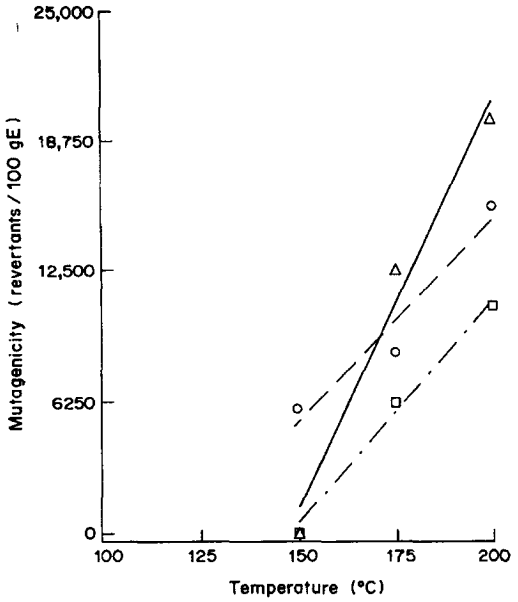

Fig. 2. Linear relationships between mutagenicity and frying temperature for samples of meat (□; $n = 3$), heart (○; $n = 3$) and tongue (△; $n = 3$).

demonstrated for all three muscles fried at 175 or 200°C, at between 6000 and 19,600 revertants/100 gE.

Figure 2 shows linear relationships obtained between mutagenicity (revertants/100 gE) and temperature for each of the samples of meat, heart and tongue. For liver and kidney, no significant mutagenicity was produced at any of the three frying temperatures.

Colour values varied between 0.9 and 6.6 (absorbance/gdm), the highest values being for meat and liver. A slight increase in colour with frying temperature was evident for the meat and tongue crust but not for the patties from the other three tissues.

The variables monitored in the crust of the 15 sets of patties fried at various temperatures (150, 175 and 200°C; Table 4) were subjected to stepwise multiple regression analyses. First the variation in mutagenicity, expressed in revertants/gdm was evaluated against six variables—creatine, creatinine, creatin(in)e, ratio of creatine/creatin(in)e, temperature and coloration. The calculations were performed on variables expressed per gdm. Among the subsets based on one variable, the creatinine concentration explained most—74% of the mutagenic variation. Next came creatin(in)e accounting for 61%, while creatine accounted for 17%. Subsets of two variables gave the highest degree of correlation with creatine and creatinine (95%). All six variables together accounted for 96% of the mutagenic variation in the crust.

A corresponding evaluation of the variation in colour development in the crusts revealed very low degrees of correlation with temperature, mutagenicity, creatine or creatinine—usually below 30% regardless of subsets of one, two or more variables.

Fried patties made of mixtures of meat with heart, tongue, liver or kidney

Table 1 shows the design of frying experiment 2 and processing data on weight loss, patty and crust thickness and yield of crust. The weight losses were almost the same for all mixtures (about 50%). Crust thickness was 1 mm on each side for most samples and the total yield of crust varied between 5 and 10 gdm/100 gE.

Table 5 shows the contents of creatine and creatinine, the mutagenicity and the colour values for these patties. As the meat content of the mixtures decreased, the creatin(in)e levels also decreased. The ratio of creatinine/creatin(in)e varied between 42 and 66% in these mixtures.

All the mutagenicity data shown in Table 5 were calculated from dose-response curves. The mutagenicity of the mixtures of meat/heart and meat/tongue varied between 10,800 and 17,300 revertants/100 gE. In the mixtures of meat/liver and meat/kidney the mutagenicity (revertants/100 gE) decreased with the decreasing proportion of meat.

Figure 3 shows the linear relationships for the meat/liver and meat/kidney samples where mutagenicity is related to the proportions of meat and offal in the mixture (100/0, 90/10, 70/30, 60/40 and 0/100). The values for slopes and intercepts of the two lines were almost equal.

In order to evaluate the influence of the calculated contents of precursors of raw patties on the variation

Table 5. Frying experiment 2: amounts of creatine and creatinine, mutagenicity data and colour values for crusts of mixtures of ground meat and other bovine tissues fried for 3 min at 200°C on a double-sided Teflon-coated plate

Source of sample	Mixture (%)	Creatine (μmol/gdm)	Creatinine (μmol/gdm)	Creatin(in)e* (μmol/gdm)	Creatinine/ creatin(in)e (%)	Mutagenicity (no. of revertants) No./gdm	Mutagenicity (no. of revertants) No./100 gE	Coloration (Abs/gdm)
Meat/heart	90/10	68	85	153	56	1759	12537	6.8
	75/25	58	82	140	59	2044	15207	7.1
	60/40	49	79	128	62	2201	10785	6.8
Meat/tongue	90/10	56	79	135	59	2283	17123	6.8
	75/25	46	64	110	58	2507	17048	6.8
	60/40	33	64	97	66	2400	17280	6.2
Meat/liver	90/10	60	67	127	53	1083	13208	7.1
	75/25	45	44	89	49	1193	10400	6.9
	60/40	37	27	64	42	464	5262	6.4
Meat/kidney	90/10	56	75	131	57	829	8217	6.9
	75/25	51	55	106	52	1435	8509	5.9
	60/40	43	50	93	54	863	5657	5.5

gdm = grams dry matter gE = grams initial raw weight

*Creatin(in)e = creatine plus creatinine.

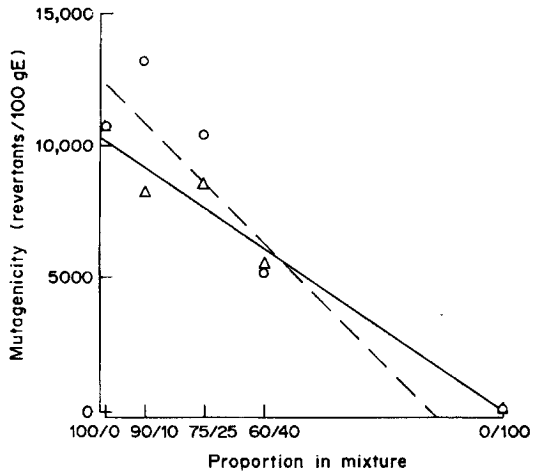

Fig. 3. Linear relationships between mutagenicity and the proportion of meat to other tissue for mixtures of meat/liver (○; $n = 5$) and of meat/kidney (△; $n = 5$). The equations were defined as $y = -125x + 12315$ (---) and $y = -102x + 10196$ (——) and the correlation coefficients as −0.94 and −0.98, respectively.

of mutagenicity produced in the crust, stepwise multiple regression analyses were performed on 17 samples of single-tissue and mixed patties fried at 200°C (Table 4 & 5). All calculations were based on variables expressed in amounts per 100 gE. Among subsets based on one variable, the creatin(in)e value in the raw patties showed the highest degree of correlation (75%). Subsets of two variables showed creatine together with creatin(in)e to account for the highest percentage of the mutagenic variation (89%). Next came the monosaccharides together with free amino acids, which accounted for 83% of the mutagenic variation of the crust. Stepwise multiple regression analyses were also performed on the variables that were monitored in the crust ($n = 17$; temperature 200°C; Tables 4 & 5) against five variables: creatine, creatinine, creatin(in)e ratio of creatinine/creatin(in)e and coloration. These calculations were performed on variables expressed per gdm. Subsets of one variable showed the highest degreee of correlation of mutagenic variation with creatinine (64%), while creatine accounted for 34%. The highest degree of correlation of mutagenic variation with two variables was found with creatine and creatinine, amounting to 73%. All five variables together accounted for 76% of the mutagenic variations in the crust.

The colour values varied between 5 and 7 (absorbance/gdm) with small but inconsistent variations (Table 5). As discussed above, multiple regression analyses of the variation of coloration in the crust were first evaluated using the same 17 samples and the variables monitored in the raw patties such as free amino acids, total monosaccharides and creatine and creatin(in)e on the basis of amounts per 100 gE. Among the subsets of single variables, total monosaccharides and free amino acids accounted for 80 and 72%, respectively. Subsets of two variables accounted for as much as 87% of the colour variation in the case of monosaccharides together with free amino acids. Stepwise multiple regression analyses applied to variables monitored only in the crust in order to evaluate variations of colour development showed very low degrees of correlation with mutagenicity, creatine and creatinine concentrations.

DISCUSSION

Frying meat patties commercially in Sweden is usually performed with patties about 10 mm thick, an equipment temperature of about 180°C and a cooking time of 3–5 min. This procedure usually results in a weight loss of 20–30%. The conditions for the frying experiments described here could thus be considered as being close to normal conditions, although the weight losses were higher (45–70%), probably because of the effective heat transfer achieved with the double-sided Teflon-coated equipment. In Sweden, only fried meat and liver are used for food, but the five tissues—meat, heart, tongue, liver and kidney—were chosen as examples of bovine tissues containing different levels of precursors of potential mutagens.

Comparing the concentrations of the different low-molecular-weight compounds in raw tissues, the contents of creatine, total free amino acids and dipeptides in meat were almost equimolar, at about 20–30 μmol/g wet tissue. This was twice the content of monosaccharides. In heart and tongue the levels of creatine and total free amino acids were much higher than those of dipeptides and monosaccharides in the same tissue. In liver, the levels of glucose and total free amino acids were very much higher than those of the other two low-molecular-weight groups (creatin(in)e and dipeptides). In the kidney, only the levels of total free amino acids were high.

The mutagenicity was related to frying temperature for each of the tissues (Fig. 2). For meat, this finding is in accordance with other studies in which the temperature was varied (Dolara *et al.* 1979; Felton & Hatch, 1986; Pariza *et al.* 1979; Spingarn & Weisburger, 1979). The value we obtained with meat fried at 200°C—10,800 revertants/100 gE—is similar to the values reported by others, 6800–23,500 revertants/100 gE for beef patties fried for 5–12 min and assayed in Salmonella strain TA98 with S-9 mix (Bjeldanes *et al.* 1982a; Commoner *et al.* 1978). Most pan-frying studies reported in the literature have been performed in the temperature range 200–300°C rather than 150–200°C.

Knize *et al.* (1985) determined by high-pressure liquid chromatography the amounts of three different IQ-type compounds and three unknown compounds in ground beef of various thicknesses (5 and 15 mm) fried at 200 or 300°C. They found that the distribution of mutagenic peaks was independent of the thickness as well as of cooking temperature. This could explain the fact that we found a linear relationship between mutagenicity and temperature (Fig. 2), although it is known that the different IQ compounds all have different specific mutagenic activities (Felton *et al.* 1986).

The heart and tongue samples showed higher mutagenicity (revertants/100 gE) at all three temperatures than the meat sample (Table 4). The heart samples showed the highest mutagenicity even though the crust was only peeled from the meat

samples. It was previously shown that a higher fat content in minced meat resulted in a shorter time being required to reach a fixed meat-surface temperature (Holtz *et al.* 1985), because fat is an effective heat-transfer agent. This means that in the frying, for example, of tongue (12% fat) and meat (3% fat) at 200° for 3 min, as in this study, the surface temperature of tongue would have been reached faster because of the higher fat content. The mutagenicity value would thus be expected to be higher since the production of mutagenicity is temperature dependent. However, this phenomenon does not explain the finding that the mutagenicity of the heart sample (with 2% fat in raw tissue) was higher than that of the meat sample (3% fat).

Bjeldanes *et al.* (1982b) investigated different organs for mutagenicity. In their study, the boiling of tongue at 100°C did not produce any significant mutagenicity. Heart, when baked, produced low mutagenicity but braising gave higher values, 830 revertants/100 gE in strain TA1538. The samples were cooked whole by Bjeldanes *et al.*, while our samples were minced. The thicknesses were the same.

Liver and kidney did not show any significant mutagenicity after frying at any of the three temperatures up to 200°C in our study. Bjeldanes *et al.* (1982b) studied the cooking of liver and kidney (not minced but coated with flour). After pan-frying liver for 3 min on each side at 300°C there was no significant mutagenicity while 4.5 min per side gave 210 revertants/100 gE. Sautéed kidney (7 min at 450°C) gave 830 revertants/100 gE (TA1538). It may seem inconsistent that patties of liver or kidney did not show any significant mutagenicity in our study while Bjeldanes *et al.* (1982b) found mutagenicity in those tissues. The explanation for this is probably the cooking and boiling methods used, as our conditions were the less severe. No data on the glucose or creatine contents of the liver and kidney were presented by Bjeldanes *et al.* (1982b). Although low in creatin(in)e, both liver and kidney may have contained sufficient amounts for the production of small quantities of mutagenicity in their study.

From Fig. 3, it is evident that pure samples together with mixtures of meat with liver or kidney showed lines with almost equal slopes and intercepts. Mixing meat with either liver or kidney decreased the creatine content and increased the free amino acid content of the samples. However, the glucose content increased for the meat/liver mixture but not for the meat/kidney mixture (Table 2). As previously reported (and shown in Table 3), threonine, glycine, lysine, alanine and serine are the five amino acids that produced mutagenicity most readily in a model system when heated together with glucose and creatine (Jägerstad *et al.* 1983b).

It is known that the creatine content of raw tissues varies between muscles, organs and offal but that the levels in the same type of tissue from cattle, pigs and other species are similar (Olsman & Slump, 1981). It is also known that the activity of a muscle is related to the amounts of red and white fibres, as reflected in the terminology of red and white muscle (Lawrie, 1979). White fibre has the more rapid energy metabolism, and it could be supposed that the creatine content is greater in white than in red fibre (Miller, 1985). Bjeldanes *et al.* (1982b) indicated that white chicken meat was more mutagenic than red chicken meat. The relationship between the creatine content of red and white muscle fibre (meat) and mutagenicity after cooking needs further study.

In our study it was evident that creatine was converted to creatinine with increasing temperature (Fig. 1). This is in accordance with Snider & Baldwin (1981), who found, by analysing whole steak, that about 13% of creatine was converted when beef was ordinarily cooked. These authors also found an approximate 20% loss of creatin(in)e during cooking. A tendency to lose creatine and creatinine during cooking was also indicated in our study. This may have been due either to the chemical reactivity of creatine and/or creatinine with other compounds or to drip losses. It was previously shown that after the baking of beef steaks, higher levels of creatine and creatinine were found in gravies, indicating drip losses (Laser Reuterswärd *et al.* 1987).

Our main purpose was to study the influence of the precursors creatine and creatinine, monosaccharides and free amino acids on the development of mutagenicity. Results of the stepwise multiple regression analyses applied to these precursors in raw patties put the concentration of creatin(in)e in first position. It was not possible to draw a clear distinction between the effects of creatine and of creatinine in the raw patties since creatinine constituted less than 10% of the total concentration in the three muscles. Among the variables monitored in the crust of the fried patties, the creatinine concentration explained more of the mutagenic variation than did the creatine concentration.

In raw meat, the dipeptide carnosine (β-alanine and histidine) was found in a concentration almost equimolar to the total amounts of free amino acids and creatine. In the other tissues the concentrations of dipeptides were very low. In an unpublished study, carnosine or β-alanine were each heated (at 128°C for 2 hr) with creatinine and glucose in a model experiment as described by Jägerstad *et al.* (1983b). Each compound yielded a mutagenic activity of about 60 revertants/μmol amino acid. This value is about the same as those demonstrated for some free amino acids in the same model system, as described in our previous study (Jägerstad *et al.* 1983b). The data thus show that a dipeptide can be reactive in this kind of system either as an intact dipeptide or following cleavage to β-alanine (and histidine) during boiling. However, the probable contribution of carnosine to mutagenicity in fried meat would be quite small, since there seems to be a surplus of free amino groups in relation to creatine or creatinine in bovine tissues.

Since the Maillard reaction seems to be involved in the mutagenic activity of cooked meat, we expected the coloration that developed to be a crude indication of the mutagenicity produced. However, no significant correlation between mutagenicity and coloration could be demonstrated. Stepwise multiple correlation analyses indicated that monosaccharides and amino acids together explained most of the colour variation. Thus liver, with its extremely high content of both sugar and free amino acids, produced the highest colour value after frying. The fact that these typical precursors of the non-enzymatic brown-

ing reaction (Maillard reaction) varied to such a great extent in the various bovine tissues that were compared in the present study may explain why colour development had such a small influence on the mutagenicity produced. Only when meat samples of the same origin are cooked under various conditions does the mutagenicity produced seem to correlate significantly with the colour development (Commoner *et al.* 1978; Holtz *et al.* 1985; Jägerstad *et al.* 1983a; Laser Reuterswärd *et al.* 1987).

In conclusion, the study reported here clearly shows that among the three groups of precursors (creatine or creatinine, monosaccharides and free amino acids) known to be required for producing IQ-compounds in model systems, the creatine or creatinine seems to be the most important factor in the production of mutagenicity in fried patties of meat, tongue, heart, liver and kidney. However, monosaccharides and free amino acids were shown to be the most important precursors for colour development in fried patties. In crusts, the concentration of creatinine was the variable determining most of the mutagenicity. In a previous study, however, it was also shown that if the glucose value in meat was extremely low, no mutagenicity was produced (Jägerstad *et al.* 1983a). The study reported here suggests that controlling the conversion of creatine to creatinine during cooking may be a way of limiting the formation of mutagens.

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The therapeutic role of creatine in Huntington's disease

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Abstract

Huntington's disease (HD) is an autosomal dominant and fatal neurological disorder characterized by a clinical triad of progressive choreiform movements, psychiatric symptoms, and cognitive decline. HD is caused by an expanded trinucleotide CAG repeat in the gene coding for the protein huntingtin. No proven treatment to prevent the onset or to delay the progression of HD currently exists. While a direct causative pathway from the gene mutation to the selective neostriatal neurodegeneration remains unclear, it has been hypothesized that interactions of the mutant huntingtin protein or its fragments may result in a number of interrelated pathogenic mechanisms triggering a cascade of molecular events that lead to the untimely neuronal death observed in HD. One putative pathological mechanism reported to play a prominent role in the pathogenesis of HD is mitochondrial dysfunction and the subsequent reduction of cellular energy. Indeed, if mitochondrial impairment and reduced energy stores play roles in the neuronal loss in HD, then a therapeutic strategy that buffers intracellular energy levels may ameliorate the neurodegenerative process. Sustained ATP levels may have both direct and indirect importance in ameliorating the severity of many of the pathogenic mechanisms associated with HD. Creatine, a guanidino compound produced endogenously and acquired exogenously through diet, is a critical component in maintaining much needed cellular energy. As such, creatine is one of a number of ergogens that may provide a relatively safe and immediately available therapeutic strategy to HD patients that may be the cornerstone of a combined treatment necessary to delay the relentless progression of HD.

Keywords: Huntington's disease; Therapy; Bioenergetics; Creatine; Mitochondrial dysfunction; Triplet repeat disorders

Abbreviations: 3-NP, 3-nitropropionic acid; ALS, amyotrophic lateral sclerosis; HD, Huntington's disease; PCr, phosphocreatine; MRS, magnetic resonance spectroscopy; NMDA, *N*-methyl-D-aspartate; UHDRS, Unified Huntington's Disease Rating Scale.

Contents

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1. Introduction: etiology and pathophysiology of Huntington's disease

Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disease of midlife onset resulting from an expanded DNA segment containing a polymorphic trinucleotide CAG repeat that encodes the protein huntingtin (Huntington's Disease Collaborative Research Group, 1993). HD has a frequency as great as 10 cases per 100,000 and a 1–3% new mutation rate (Myers et al., 1993). HD occurs worldwide in all races and ethnic groups (Kremer et al., 1994). The frequency is lowest in Japan and highest in Venezuela along the shores of Lake Maracaibo. In the United States, there are ~30,000 HD patients and another 150,000 at genetic risk. The number of polyglutamine repeats range from 17 to 29 in the normal population. Individuals become symptomatic for HD once the repeat expands to more than 38 CAG repeats. There is an inverse relationship between age of onset and CAG repeat number, in which younger age onset correlates with higher repeat number. Although a number of hypothetical pathologic mechanisms have been suggested since the discovery of the gene, a direct pathway from the genetic mutation to neuronal degeneration has not been established. Huntingtin is a widely expressed cytoplasmic protein found heterogeneously in neurons throughout the brain. Although huntingtin's function remains unknown, involvement in intracellular transport, autophagy, transcription, mitochondrial function, and signal transduction have been postulated based on its protein–protein interactions. Mutant huntingtin has been reported to inhibit fast axonal transport and destabilize microtubules within the cell (Szebenyi et al., 2003; Trushina et al., 2004). In HD, both normal and mutant alleles are expressed and both gain of function alterations in which mutant huntingtin protein is toxic, as well as a loss of function of the normal protein, may play roles in the pathogenesis of the disease (Ross, 1997; Nucifora et al., 2001; Cattaneo, 2003; Zhang et al., 2003; Ross & Poirier, 2004). Although there has been significant progress in understanding the disease process in the subsequent 130 plus years since Huntington's description, there is currently no treatment to prevent the onset or to delay the insidious and relentless course of HD.

The initial detailed description of HD was that of George Huntington, a medical practitioner from Pomeroy, OH, in 1872 (Huntington, 1872). He gave a thorough account of the disease based upon the clinical descriptions taken by his father and grandfather from their practice in East Hampton, Long Island, NY. These patients could be traced to a few individuals that had emigrated from a small village in Suffolk, England, from 1630 to 1649 (Vessie, 1932). While initially thought to be a chronic encephalitis, in 1908, Jergelsma first described the characteristic neuropathological alterations within the basal ganglia that are now accepted as the *sine qua non* of HD (Jergelsma, 1908; Bruyn et al., 1979). While other brain regions are involved, the most striking neuropathological changes are gross atrophy of the neostriatal nuclei, the caudate nucleus, and putamen, with concomitant marked neuronal loss and astrogliosis (Vonsattel et al., 1985; Kowall & Ferrante, 1998; Hersch et al., 2004; Fig. 1). Not all striatal neurons are effected equally, such that there is a selective pattern of neuronal vulnerability and topographic susceptibility in which intrinsic locally arborizng aspiny striatal interneurons are relatively spared, while medium-sized striatal spiny projection neurons are affected earliest and most severely (Fig. 2; Ferrante et al., 1985, 1986, 1987, 1991; Graveland et al., 1985; Kowall et al., 1987; Hersch et al., 2004).

There is increasing evidence to suggest that mutant huntingtin and its proteolytic fragments may participate in pathologic protein–protein interactions, leading to altered genetic and molecular messages that result in dysfunctional neurons which are then susceptible to further generic stresses and eventual cell death (Beal & Ferrante, 2004). Among the pathogenic mechanisms, these interactions may trigger transcriptional dysregulation, mitochondrial dysfunction, proapoptotic signaling, oxidative injury, excitotoxicity, inflammatory reactions, and malfunctioning proteolysis. These pathologic messages can interact and potentiate one another. In the context of this review, energy depletion can exacerbate many of these pathologic processes. Energy is critical to the biological and molecular regulation of multicellular functions, and as such, reduced energy levels threaten cellular homeostasis and integrity. Spiny striatal neurons may be selectively involved because of a combination of their position within the brain circuitry and their intensive energy needs for maintaining unusually high membrane potentials (Olney & de Gubareff, 1978a; Whetsell & Schwarcz, 1983; Graveland et al., 1985; Ferrante et al., 1991; Sawa, 2001; Brustovetsky et al., 2003; Rego & Oliveira, 2003; Galas et al., 2004).

The concept of defective cellular energy metabolism in neurological diseases, particularly HD, was suggested by Albin and Greenamyer as an alternative excitotoxic hypoth-

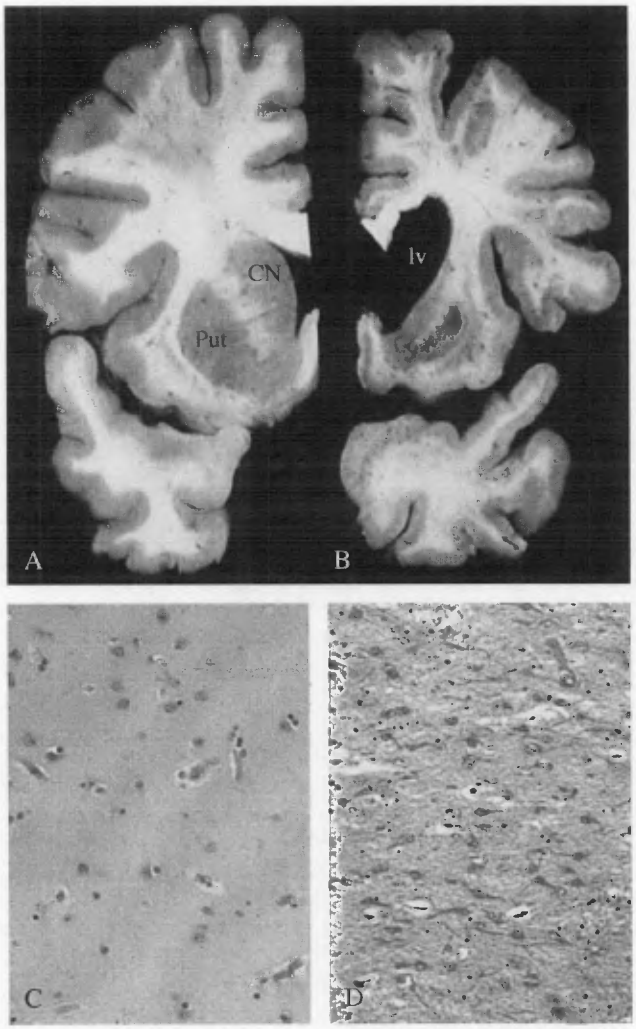

Fig. 1. Gross and microscopic neuropathology in Huntington's disease. Coronal sections from formaldehyde-fixed cerebral hemispheres at the level of the head of the caudate nucleus (CN) and putamen (Put) from an age-matched normal control (**A**) and a 62-year-old female with Huntington's disease (**B**). Note the marked gross atrophy of the neostriatum (CN and Put) and enlarged lateral ventricle (lv) in Panel **B**. The corresponding hematoxylin and eosin staining of the caudate nucleus shows significant neuronal loss and astrogliosis in Huntington's disease (**D**), in comparison with the normal control (**C**).

esis and has been coined 'slow onset excitotoxicity' (Albin & Greenamyre, 1992; Beal, 1992). The relevant observations were first made by Olney, showing that partial membrane depolarization produced *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity, in which the voltage-dependent magnesium block was released from the NMDA calcium channel (Olney & de Gubareff, 1978a, 1978b; Novelli et al., 1988; Zeevalk & Nicklas, 1991). Strong evidence exists for early metabolic dysfunction, energy depletion, and a role of increased oxidative signaling in patients with HD. Weight loss occurs early, frequently prior to the onset of the movement disorder (Djousse et al., 2002). Positron emission tomography studies have demonstrated reduced glucose utilization in both presymptomatic and symptomatic HD patients (Kuhl et al., 1985; Mazziotta et al., 1987; Kuwert et al., 1990). Glucose hypometabolism appears early, prior to striatal atrophy (Kuhl et al., 1982, 1985). Magnetic resonance spectroscopy (MRS) has demonstrated a significant decrease in the phosphocreatine (PCr)-to-inorganic phosphate ratio in resting muscle in patients with HD and increased lactate concentrations in the cerebral cortex (Koroshetz et al., 1997). In another study, increased lactate levels were reported in both the basal ganglia and occipital cortex in symptomatic HD patients, however, augmented lactate levels were not present in HD patients asymptomatic or at risk for the disease (Jenkins et al., 1993, 1998). These findings suggest that there is a progression of impaired energy metabolism. Energy defects may result from mitochondrial damage caused by oxidative stress as a consequence of free radical generation. Ultrastructural studies on brain biopsies of HD patients have provided evidence of mitochondrial abnormalities and increased lipofuscin, a pigment that accumulates as a consequence of free radical mediated membrane damage (Tellez-Nagel et al., 1974). In addition to exacerbated lipofuscin, evidence for oxidative damage in HD also includes DNA fragmentation and increased oxidative damage products of protein nitration, lipid peroxidation, DNA oxidation, and inducible markers for oxidative stress (Browne et al., 1999). Consistent with the findings of mitochondrial damage, we have preliminary data that show a significant reduction in mitochondrial size and number in striatal caudate neurons in presymptomatic HD patients, with greater loss of mitochondria and reduced mitochondrial size

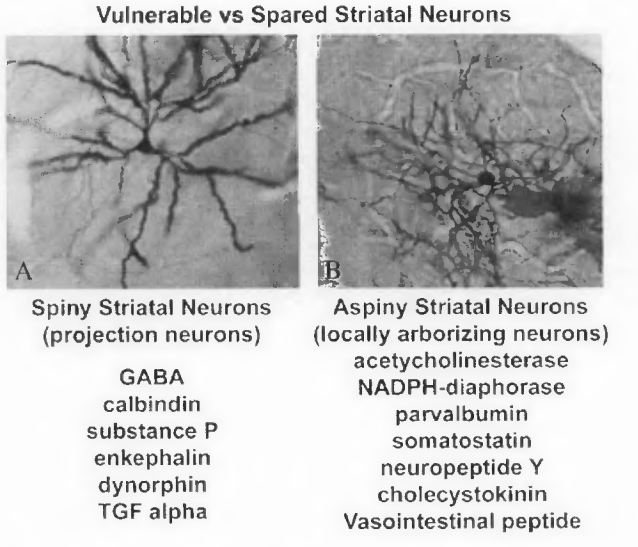

Fig. 2. Selective vulnerability of striatal neurons. Spiny striatal neurons (**A**), so named by their Golgi stained appearance of spines along their dendritic arbors, are the most plentiful striatal neuron and project outside of the neostriatum. They receive the bulk of excitatory glutamatergic cortical input to the neostriatum. As a consequence, a long held hypothesis has been that this striatal neuron subtype is subject to excitatory injury. These neurons, and their neurochemical substances, are most vulnerable to the degenerative process. (**B**) Aspiny neurons, so named by their relative lack of spines on their dendritic arbors, and their associated neurochemical substances are relatively spared in Huntington's disease.

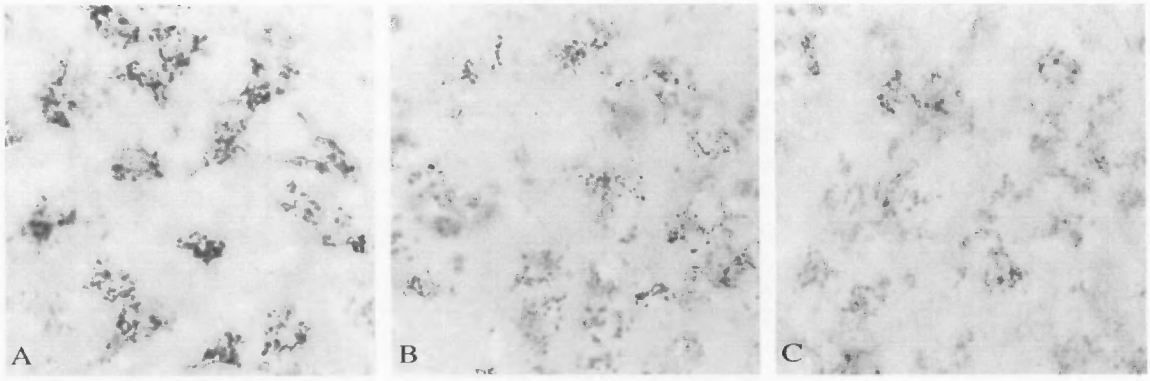

Fig. 3. Mitochondrial abnormalities in Huntington's disease. Immunocytological localization of mitochondria in striatal caudate nucleus neurons from a normal healthy individual (**A**), a presymptomatic Huntington's disease patient (**B**), and severely symptomatic Huntington's disease patient (**C**). Note the mitochondria immunostain as round-oval puncta delineating cells within the caudate nucleus, using an anti-human mitochondria antibody from Leinco Technologies, Inc. The mitochondrial number and size are clearly reduced in the presymptomatic Huntington's disease patient and further affected in the symptomatic Huntington's disease patient, suggesting mitochondrial dysfunction and reduced energetics.

in moderate to severe grades of HD (Fig. 3). Mitochondrial dysfunction may be the result of altered electron transport activities. Indeed, while significant reductions in Complex I, II–III, and IV activities are present in the neostriatum from HD patients, they were not found in other brain regions (Brennan et al., 1985; Mann et al., 1990; Parker et al., 1990; Browne & Beal, 1994; Gu et al., 1996). Electron transport chain complex subunits have been reported to be involved in selective degeneration of the basal ganglia in Leber's optic neuropathy (Howell et al., 1991; Jun et al., 1994), and as such, it is not unreasonable to suggest that the genetic HD mutation may alter nuclear encoded components of electron transport complexes, resulting in a primary bioenergetic defect. It is of interest to note that in patients with other trinucleotide repeat diseases, such as spinocerebellar ataxias, mitochondrial abnormalities and metabolic defects are present, linking a common mechanism of energy deficiency to the polyglutamine gene mutation (Mastrogiacomo et al., 1996; Matsuishi et al., 1996). Therefore, therapeutic strategies that buffer intracellular energy levels may play

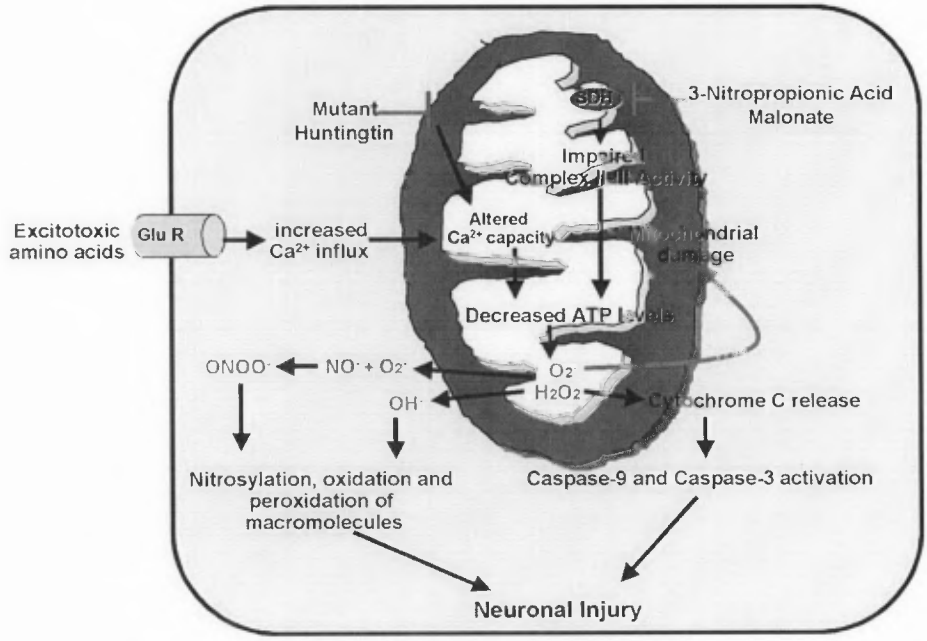

Fig. 4. Mitochondria-mediated neuronal dysfunction in HD. Mutant huntingtin proteolysis and aggregation may disrupt the mitochondrial membrane potential and alter excitotoxin-induced Ca^{2+} influx. In experimental models, mitochondrial toxins (3-nitropropionic acid and malonate) impair respiratory enzyme activities and ATP generation and increase reactive oxygen species (ROSs). This is consistent with impaired electron transport chain activities in Huntington's disease. Superoxide radical (O_2^-) interacts with nitric oxide (NO) and produces peroxinitrite ($ONOO^-$). Both ROSs and peroxinitrite may then damage cellular macromolecules through nitosylation, oxidation, and peroxidation, which can directly contribute to neuronal injury. In addition, the release of cytochrome *c* from damaged/dysfunctional mitochondria triggers the activation of apoptotic cascade and release of initiator and executioner caspases (caspase −9 and −3), resulting in neuronal cell death.

an important role in the treatment of HD and polyglutamine diseases in general.

There is also substantial evidence in experimental models of HD suggesting an important interplay between energy metabolism defects, aberrant mitochondrial function, and excitotoxicity in the pathogenesis of HD (Beal et al., 1993; Brouillet et al., 1995; Schulz et al., 1995; Beal, 1996, 2000; Palfi et al., 1996; Browne et al., 1999; Grunewald & Beal, 1999; Tarnopolsky & Beal, 2001; Browne & Beal, 2004). Both necrotic and apoptotic cell death may be triggered by reduced cellular energy (Green & Reed, 1998; Desagher & Martinou, 2000; Roy & Nicholson, 2000). Other possible sequelae of energy deficiency and mitochondrial dysfunction include reduced redox potentials of cellular membranes, dysfunction of the mitochondrial permeability transition pore, and activation of initiator and executioner caspases, each one of which may further contribute to the cell death cascade (Fig. 4; Green & Reed, 1998; Beal, 2000; Kiechle et al., 2002). Indeed, in vitro studies show that N-terminal huntingtin fragments may directly impair mitochondrial function resulting in calcium abnormalities (Panov et al., 2002) and subsequent energy deficiency. Consistent with energy depletion and the pathological phenotype of HD, there are a number of mitochondrial inhibitors that act at complexes of the electron transport chain, resulting in energy deficiency and reduced cellular levels of ATP, mimicking the behavioral and neuropathological phenotype of HD in both primates and rodents (Alston et al., 1977; Beal et al., 1993; Brouillet et al., 1993, 1995; Henshaw et al., 1994; Schulz et al., 1995; Palfi et al., 1996). One such naturally occurring plant toxin, 3-nitropropionic acid (3-NP), is an irreversible inhibitor of succinate dehydrogenase and both the Krebs cycle and Complex II activity of the electron transport chain (Candlish et al., 1969; Ludolph et al., 1991). 3-NP is associated with HD-like symptoms in both humans and animals, and as such, has been used as an experimental model for HD (Alston et al., 1977; Ludolph et al., 1991, 1992; Beal et al., 1993; Brouillet et al., 1995; Palfi et al., 1996). Accidental ingestion of 3-NP in humans results in dystonia with jerk-like movements and bilateral damage to the basal ganglia, as determined by brain imaging (Ludolph et al., 1991). In animals, experimental striatal lesions are associated with energy deficiency, showing marked reductions in cellular levels of ATP (Hamilton & Gould, 1987; Ludolph et al., 1992). Sodium azide, a Complex IV (cytochrome oxidase) inhibitor, produces striatal damage and a hyperkinetic movement disorder in primates (Mettler, 1972).

2. Creatine

Creatine is a naturally occurring compound that, through its intermediate phosphocreatine, provides a necessary cellular reserve of high-energy phosphates. While creatine may directly improve bioenergetic defects, it may also benefit other pathophysiological mechanisms associated with HD (Mallouk et al., 1999; Dzeja & Terzic, 2003; Tsim et al., 2003; Trushina et al., 2004; Verger & Crossley, 2004; Wegele et al., 2004; Belotserkovskaya et al., 2004; Bender et al., 2005; Fig. 5). Interest in the use of oral creatine supplementation began in earnest in the 1990s, as it became a widely used ergogenic supplement for performance enhancement by professional and amateur athletes (Harris et al., 1992; Tarnopolsky et al., 1992; Greenhaff et al., 1993; Wyss & Kaddurah-Daouk, 2000; Persky & Brazeau, 2001). Creatine (methylguanidino–acetic acid) is a guanidino compound synthesized endogenously from arginine, methionine, and glycine, predominantly in the liver, as well as in the kidneys, pancreas, testes (Block & Schoenheimer, 1941; Walker, 1979, Persky & Brazeau, 2001), and the brain (Braissant et al., 2001). Creatine is also derived exogenously through the diet in the consumption of meat and fish (Balsom et al., 1994). In order to maintain sufficient body stores of creatine, ~2 g is required daily through both diet and endogenous synthesis (Casey & Greenhaff, 2000). Over 90% of creatine is found in skeletal muscle, mostly as phosphocreatine (PCr), with the remaining stores in the brain and other organs (Walker, 1979; Wyss & Kaddurah-Daouk, 2000). Creatine is degraded to creatinine and excreted through the kidneys (Casey & Greenhaff, 2000). Creatine is an excellent stimulant of mitochondrial respiration, resulting in the generation of PCr (Kernec et al., 1996; O'Gorman et al., 1996). It is a critical component of the creatine kinase system in maintaining needed cellular energy. The major source of energy in the brain is ATP, which is tightly coupled to creatine and PCr levels within the cell. Creatine is shuttled across membranes via a sodium-dependent creatine transporter protein, CreaT (Schloss et al., 1994; Willott et al., 1999; Snow & Murphy, 2001), that regulates tissue levels in response to low dietary intake or high endogenous creatine levels (Guerrero-Ontiveros & Wallimann, 1998; Loike et al., 1988). Creatine kinase catalyzes the reversible transfer of a phosphoryl group from PCr to ADP, forming ATP. As such, creatine offsets energy depletion by forming PCr, providing a spatial energy buffer to re-phosphorylate adenosine diphosphate to adenosine triphosphate at cellular sites of needed energy consumption and in the reversible reaction forming PCr and ADP from creatine and ATP (PCr shuttle hypothesis; Van Brussel et al., 1983; Meyer et al., 1984; Bessman & Carpenter, 1985; Tombes & Shapiro, 1985). Augmenting creatine levels in HD may therefore help to prevent reduced energy stores and improve neuronal function. Creatine is also involved in regulating glycolysis, stabilizing the mitochondrial form of creatine kinase, and inhibiting the mitochondrial permeability transition pore (O'Gorman et al., 1996, 1997). Another potential neuroprotective mechanism of creatine supplementation is the ability of PCr to stimulate synaptic glutamate uptake and thereby reduce extracellular gluta-

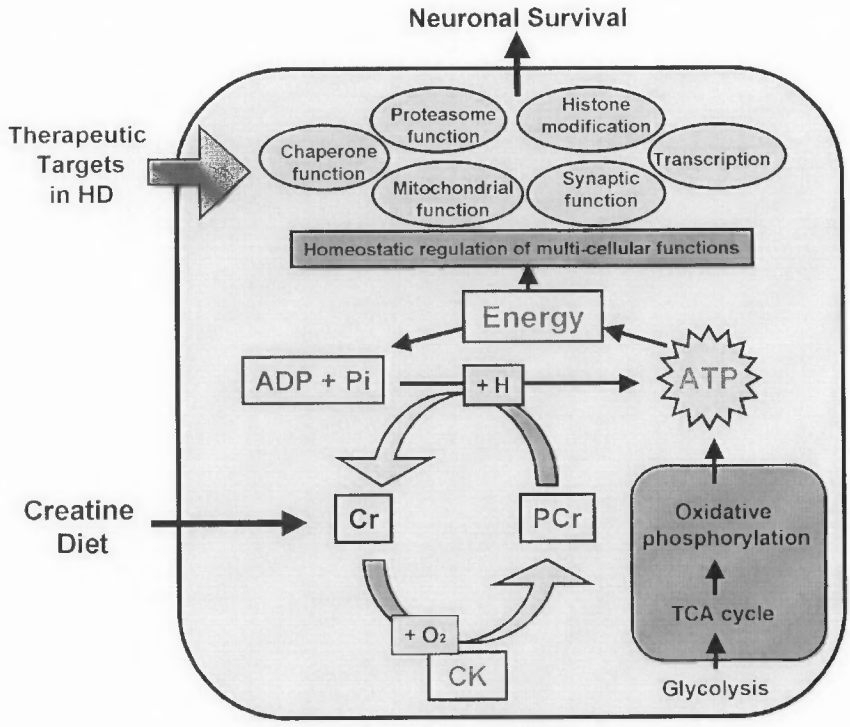

Fig. 5. Creatine (Cr) supplementation improves multicellular functions and neuronal survival. Creatine kinase (CK) catalyzes the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, to generate ATP. Phosphocreatine buffers cellular energy by connecting sites of energy production with sites of energy consumption. Energy stores are critical to the homeostatic regulation of the cell and improve hypothetical mechanisms of cellular dysfunction in Huntington's disease. Consequently, sustaining high energy ATP helps to modulate the chaperone-ATPase function and activate ATP-dependent ubiquitin–proteasome function. Energy-dependent conversion of glutamate (Glu) to glutamine (Gln) via the Glu–Gln cycle modulates synaptic activity and lowers excitotoxicity. The ATP-dependent chromatin remodeling enzyme regulates chromatin structure and counterbalances its repressive effects on transcription in neurons. Collectively, neuronal energy reserves, such as creatine, may play an important role, favoring survival in modulating many cellular activities.

mate (Xu et al., 1996). In addition, creatine has also been reported to act as an antioxidant, scavenging reactive oxygen species (Lawler et al., 2002). It has recently been suggested that the neuroprotective effects of creatine may be independent of mitochondrial creatine kinase (Klivenyi et al., 2004a, 2004b). Although animal studies provide a therapeutic rationale for creatine, more information on the pharmacokinetics of creatine in humans is needed (Harris et al., 1992; Schedel et al., 1999; Wyss & Schulze, 2002).

3. Experimental evidence for creatine supplementation in Huntington's disease

3.1. Preclinical trials in animal models of Huntington's disease

As noted above, creatine administration has several potential neuroprotective effects, including buffering of intracellular energy reserves, stabilizing intracellular calcium, reducing extracellular glutamate, inhibiting activation of the mitochondrial pore transition, and acting as an antioxidant. Along with creatine kinase and PCr, creatine constitutes an energy buffering and transport system, connecting sites of energy production with sites of energy consumption. The salubrious neuroprotective effects of creatine have been widely reported in experimental models of neurological diseases, particularly neurotoxin and transgenic models of HD (Carter et al., 1995; Matthews et al., 1998, 1999; Balestrino et al., 1999; Klivenyi et al., 1999, 2003, 2004a, 2004b; Brewer & Wallimann, 2000; Ferrante et al., 2000; Ikeda et al., 2000; Malcon et al., 2000; Shear et al., 2000; Sullivan et al., 2000; Andreassen et al., 2001a; Brustovetsky et al., 2001; Hausmann et al., 2002; Dedeoglu et al., 2003; Royes et al., 2003; Zhu et al., 2004). Using the mitochondrial toxins 3-NP and malonate to mimic the energy deficiency found in HD, creatine supplementation significantly reduced striatal lesion volumes (Matthews et al., 1998). This neuroprotection by creatine was associated with higher levels of PCr and creatine and reduced lactate levels in the brain, consistent with improved energy production. These neuroprotective findings have been confirmed in a rat 3-NP model (Shear et al., 2000).

Genetic animal models have greatly advanced the study of human neurological diseases, providing experimentally accessible systems to study molecular pathogenesis and to test potential therapeutic strategies for translation to humans experiencing these diseases. Transgenic mouse models of HD, which have been used extensively to assess potential neuroprotective therapies and have known energetic deficits in the brain, have been ideal for examining the therapeutic

potential of creatine (Beal & Ferrante, 2004; Hersch & Ferrante, 2004). While there are a number of bioenergetic therapeutic agents, including creatine, coenzyme Q10, and lipoic acid, that augment energy levels and improve the behavioral and neuropathological phenotype in transgenic HD mice (Ferrante et al., 2000; Andreassen et al., 2001b, 2001c; Ferrante et al., 2002; Dedeoglu et al., 2003), dietary creatine supplementation has had the greatest efficacy (Ferrante et al., 2000). Creatine supplementation formulated at 1%, 2%, and 3% in the diet (chow), starting at 3 weeks of age, significantly improved survival, reduced gross brain atrophy, delayed atrophy of striatal neurons, and reduced the formation of mutant huntingtin protein aggregates in both the striatum and neocortex. In addition, motor performance was improved and body weight loss was reduced in the creatine-treated R6/2 mice. The brain levels of creatine were significantly increased in the treated mice, as determined using nuclear magnetic resonance spectroscopy, while decreases in *N*-acetylaspartate (NAA) concentrations were delayed (Ferrante et al., 2000). There was an inverted 'U' shaped efficacy curve according to dose, such that 2% creatine in the diet resulted in an ~18% extension of survival in the R6/2 mice, while both 1% and 3% creatine chow formulations resulted in ~9% and 5% in survival extension, respectively. The reduced efficacy with 3% creatine may reflect down-regulation of the creatine transporter leading to a relative reduction in brain creatine levels. Creatine treatment in another transgenic model of HD, N171-82Q HD mouse model, confirmed the initial results (Andreassen et al., 2001a). The effectiveness of creatine at different stages of the R6/2 phenotype has also been examined by initiating creatine administration (2% in the chow) well after clinical symptoms appear in the R6/2 mice at 6, 8, and 10 weeks of age (Dedeoglu et al., 2003). These time points are analogous to early, middle, and late stage disease in human HD. There was a significant extension in survival in the 6- and 8-week start groups, as well as improved motor performance, body weight, and neuropathology. While there was a significant reduction in creatine and ATP in the striatum of untreated R6/2 mice, creatine and ATP levels were markedly improved by 39% and 65%, respectively, in the creatine-treated R6/2 mice. These findings are consistent with a role for energy deficiency in HD pathogenesis and suggest that creatine therapy may benefit HD patients if started before or after clinical symptoms are present. Because there have been few phase III studies in HD patients at this time, it has not yet been confirmed that experiments demonstrating improved phenotypes in transgenic mice are predictive of benefits in humans. Similarly, it is unknown whether the magnitude of benefit in mice predicts the magnitude of benefit in humans. Nevertheless, preclinical studies using creatine in HD mouse models have provided the therapeutic rationale for the use of creatine in HD patients. Positive results from human clinical trials will illuminate the value of mouse clinical trials.

4. Creatine in health and disease

4.1. Clinical studies in normal healthy individuals

Dietary creatine supplementation in normal healthy individuals has beneficial effects on muscle function, and as such, its use has dramatically increased in amateur and professional athletics over the past decade. Creatine use enhances high-intensity exercise performance, increasing muscle strength and output in both genders (Earnest et al., 1995; Vandenberghe et al., 1997; Juhn & Tarnopolsky, 1998; Maganaris & Maughan, 1998; Smith et al., 1998; Volek et al., 1999). While there are reports that creatine supplementation provides little or no benefits in the elderly (Odland et al., 1997; Bermon et al., 1998; Rawson & Clarkson, 2000; Jakobi et al., 2001; Eijnde et al., 2003), others have suggested that creatine intake is useful in preventing muscle loss and improving muscle strength and performance in this population (Chrusch et al., 2001; Wiroth et al., 2001; Gotshalk et al., 2002; Brose et al., 2003). Creatine increases fat-free mass in the setting of heavy resistance training (Volek et al., 1999). It is unclear, however, whether weight gain may, in part, be a consequence of water retention. Interestingly, the ergogenic response to creatine supplementation can vary greatly between individuals (Juhn & Tarnopolsky, 1998). It appears that there are individuals that do not respond to creatine supplementation (Greenhaff et al., 1993). Muscle biopsy samples taken from 8 normal subjects after ingestion of 20 g creatine for 5 days resulted in marked increases in total creatine and PCr in 5 individuals, but little or no effect on creatine or PCr levels in the remaining 3 subjects (Greenhaff et al., 1993). In addition, variability in tissue creatine concentrations among individuals has been described (Harris et al., 1992; Green et al., 1996). In normal healthy individuals, the continuous and prolonged use of creatine fails to keep creatine and PCr elevated in muscle (Vandenberghe et al., 1997; Volek et al., 1999; Hespel et al., 2001). Habituation to creatine intake is a well-recognized phenomenon in the sports community and, as such, has resulted in a cycling strategy in which intake is stopped and resumed after a period of time. It is unclear, however, if this practice is effective because little is known about the levels and duration of creatine supplementation for this down-regulation to occur (Snow & Murphy, 2001; Lemon, 2002). It has been shown, however, that the response to high creatine concentration results in a down-regulation of the creatine transporter (Crea T) activity and transporter protein content (Guerrero-Ontiveros & Wallimann, 1998; Loike et al., 1988; Dodd et al., 1999; Snow & Murphy, 2001; Lemon, 2002). While the mechanism by which this occurs is not understood, it is unlikely that intracellular increases alone are the causal factor. Although increased creatine levels may lead to the down-regulation of the creatine transporter in muscle, it is less clear whether this inverse relationship occurs in the brain (Kekelidze et al., 2000) and not at all understood in

disease states where creatine is deficient. The chronic long-term effects of creatine supplementation in normal healthy individuals and diseased patients remain poorly understood, as do all the factors that contribute to individual differences in physiologic responses and tissue levels of creatine (Baker & Tarnopolsky, 2003; Derave et al., 2003).

4.2. Human clinical trials in neurological disease

There have been some successes in the use of creatine supplementation in neurologic diseases, most clearly in genetic diseases associated with creatine metabolism. Both arginine:glycine amidinotransferase deficiency and guanidinoacetate methyltransferase deficiency, which are associated with a lack of creatine and PCr, are improved clinically by creatine supplementation (Stockler et al., 1996a, 1996b; Tarnopolsky & Martin, 1999; Wyss & Schulze, 2002; Sykut-Cegielska et al., 2004). Secondary disorders of creatine metabolism also respond to creatine supplementation. In gyrate atrophy, an autosomal recessive disorder causing hyperornithinemia and resulting in chorioretinal degeneration and type 2 muscle atrophy, creatine therapy increases muscle area, augments PCr/P ratios by 1.5-fold, normalizes PCr/ATP ratios, and reduces tubular aggregate abnormalities in skeletal muscle (Heinanen et al., 1999a, 1999b). These patients were treated with creatine for 8–15 years, using 1.5–2.0 g/day, evidence that prolonged creatine treatment may be possible in other disorders of creatine and PCr deficiency or dysregulation. Creatine may also improve the clinical symptoms of neurological disorders associated with energetic dysfunction, such as mitochondrial encephalopathies and in certain muscular dystrophies (Hagenfeldt et al., 1994; Kremer et al., 1994; Lofberg et al., 2001; Barisic et al., 2002; Walter et al., 2002; Komura et al., 2003; Tarnopolsky et al., 2004a, 2004b, 2004c). There is, however, less evidence for creatine benefiting other types of neuromuscular diseases, such as Charcot–Marie–Tooth (Chetlin et al., 2004) and myotonic dystrophy type 1 (Tarnopolsky et al., 2004b).

There are compelling scientific and clinical reasons suggesting that creatine supplementation might be neuroprotective in a variety of chronic neurodegenerative disorders in which secondary bioenergetic defects have been suspected (Tarnopolsky & Beal, 2001; Baker & Tarnopolsky, 2003; Browne & Beal, 2004; Ellis & Rosenfeld, 2004; Schols et al., 2005). On this basis, there have been several clinical trials in amyotrophic lateral sclerosis (ALS). A double-blind, placebo-controlled, sequential clinical trial to assess the efficacy of creatine monohydrate on survival and disease progression in ALS patients using 10 g/day did not find evidence of a beneficial effect on survival or disease progression (Groeneveld et al., 2003). In another randomized, double-blind, placebo controlled trial in 104 patients with ALS treated with 5 g/day creatine over 6 months, there was also no benefit (Shefner et al., 2004). These were pilot studies, however, with limited sample sizes in which improvements would need to have been quite large to be detected. In addition, it has been suggested that target dose using bioenergetic nutriceutical compounds may need to be much greater in treating neurodegenerative disorders (Shults et al., 2002).

4.3. Clinical trials in Huntington's disease

There is strong evidence to suggest that a bioenergetic defect exists in HD as discussed above. Creatine supplementation is intended to augment cerebral energy reserves and thereby reduce neuronal metabolic and oxidative stress and slow neurodegeneration. While there have been several clinical trials of creatine in HD, none have been powered to detect significant slowing of progression and none have revealed any improvement in clinical measures. These trials have, however, demonstrated the safety and tolerability of creatine in HD patients. In addition, the biomarkers used in these studies have provided information that supports further trials. Creatine, 3–5 g/day, has been shown to be safe and well tolerated by early-stage HD patients, with blood serum creatine levels increasing over 2-fold (Kieburtz, 2001). Verbessem and colleagues treated 26 HD patients with 5 g/day creatine and 15 patients with placebo for 1 year and found no differences in measures of strength, neurological status, or cognitive status (Verbessem et al., 2003). In a 1-year open-label pilot study, Tabrizi and colleagues treated 13 individuals with the HD genetic mutation (3 were presymptomatic 10 were symptomatic) with creatine (10 g/day) for 12 months (Tabrizi et al., 2003). Two patients were excluded from the trial, 1 for non-compliance, and the second for an abnormal rise in serum creatinine. Another 2 patients reduced their creatine dose to 5 g/day in response to diarrhea. Creatine administration in this study was safe and tolerable and resulted in increased creatine brain concentrations as demonstrated by MRS. The United Huntington's Disease Rating Scale (UHDRS) scores were unchanged after 12 months. The authors suggest, since the UHDRS did not change over the trial period, that creatine (10 g/day) may be effective in stabilizing disease progression. Two of the authors (SMH and HDR) have completed a multicenter, double-blind, placebo-controlled study of 8 g/day of creatine in 32 HD patients compared with 32 patients on placebo for 4 months. This dose was safe and tolerable and no effects on the UHDRS were observed due to the short study period. The serum levels of creatine were increased up to 15-fold. Brain levels of creatine were significantly increased by 7.2% and NAA levels (a biomarker of neuroprotection) were increased by 16% (NAA/tCr) as measured by MRS (Fig. 6). Bender and colleagues used MRS to examine another biomarker of creatine's activity in HD patients treated with 20 g/day for 5 days, followed by 6 g/day for 8–10 weeks. They demonstrated a significant reduction in glutamate levels in the parietooccipital cortex. This is very interesting because glutamate release and excitotoxicity are enhanced by energy

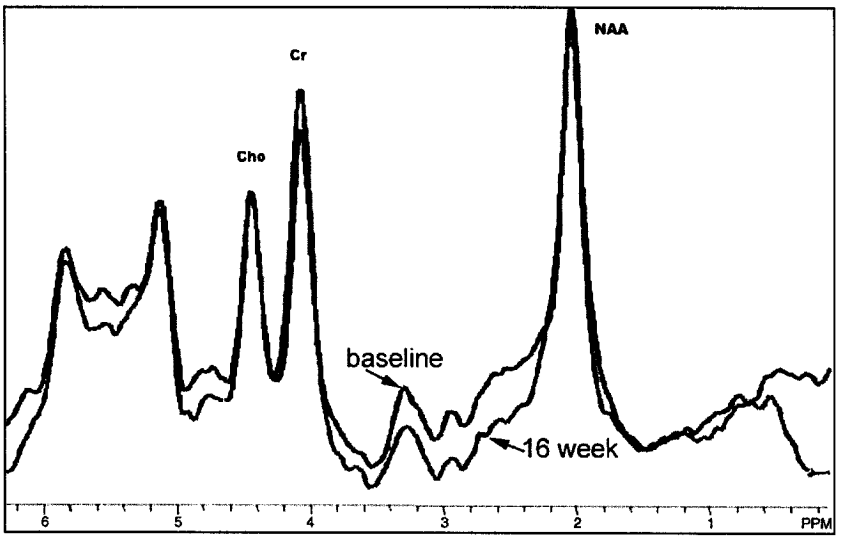

Fig. 6. Magnetic resonance spectroscopy. Spectra of the frontal cortex were obtained at baseline and 16 weeks after starting creatine in a group of early symptomatic HD subjects ($N=8$). Creatine (Cr) concentrations increased by 7.2% ($P<0.05$) and *N*-acetyl aspartate (NAA) concentrations increased by 16% ($P<0.05$) by week 16 on creatine and returned to baseline levels 4 weeks after washout. The increases in NAA may reflect measurable improvements in mitochondrial energy production and may be a sensitive marker of neuronal health.

deficiency and are considered to play a significant role in the pathogenesis of HD. None of these studies were sufficiently powered to be informative about whether or not creatine slows the clinical progression of HD, however, they do attest to its safety and tolerability and favorable effects on serum and brain levels of creatine and on biomarkers of HD pathology. The optimal dose to use in a definitive efficacy study for HD is not yet certain. The most efficacious neuroprotective dose of creatine in transgenic mouse studies was 2% of the diet, corresponding to 30–35 g/day in HD patients weighing 70 kg, suggesting that the dose of creatine supplementation in HD patients may have been underestimated. While mouse and human bioavailability may not correspond well, such a dose is at least feasible for humans. Consequently, a dose escalation study to determine whether there is a maximally tolerated dose in HD, as well as whether there are doses at which serum and brain levels of creatine are maximized, has been initiated.

5. Safety, tolerability, and adverse events of creatine

Creatine supplementation has been used in both normal healthy individuals and in patients with severe neurological disease, with some benefits, as described above. The safety of creatine has been reviewed by a number of authors (Juhn & Tarnopolsky, 1998; Persky & Brazeau, 2001; Wyss & Schulze, 2002; Baker & Tarnopolsky, 2003). There is consistency in that creatine, overall, does not have a significant deleterious effect in humans, although there is caution regarding long-term, high-dose supplementation of creatine in normal healthy individuals. There is agreement that creatine use can result in small weight gains. Creatine is a relatively safe compound that has been used as a dietary supplement for extended periods of time by athletes and in some clinical settings by patients, with few reported side effects. Some adverse events, however, have been described (Cooke et al., 1995). It has been reported that high-dose and extended use of creatine can result in elevated creatinine levels, with the potential for renal dysfunction (Mihic et al., 2000; Robinson et al., 2000; Benzi & Ceci, 2001). It has been suggested that these findings, however, may be the consequence of underlying coexisting renal disease (Pritchard & Kalra, 1998; Koshy et al., 1999). In contrast, Poortmans and others have shown that extended creatine supplementation does not alter renal function and glomerular filtration (Poortmans et al., 1997; Poortmans & Francaux, 1999; Mihic et al., 2000). Moreover, since creatinine is the primary metabolite of creatine, other measures of renal function are necessary. Creatine supplementation, however, should be closely monitored in patients at risk for renal disease. Additional side effects of creatine include mild asthmatic symptoms along with gastrointestinal distress. Muscle cramping and heat intolerance have also been reported. It is not clear, however, whether the latter is the result of poor hydration, particularly when used in sports activities. The causal events of anecdotal reports of adverse reactions to the FDA, such as rash, vomiting, anxiety, fatigue, migraine, seizures, and atrial fibrillation, are unclear and have not been scientifically substantiated. No studies in humans have suggested a significant health concern regarding creatine supplementation. Prolonged high-dose use of creatine in the sports community has been ongoing for 10–15 years, and as such, the absence of reported adverse events is important to note. No acute and subacute toxicity or mutagenicity of creatine has been shown in experimental animals (Mertschenk et al., 2001). While animal toxicology studies using creatine are lacking, we have preliminary data

in mice that 10% creatine in the diet over 4 months results in no adverse events other than minor diarrhea that can be controlled by dose escalation during the first week of supplementation. Although it has been suggested that there may be a theoretical carcinogenic effect of creatine (Wyss & Kaddurah-Daouk, 2000), it is very unlikely that the nitrosation products of creatine are present to any extent (Wyss & Schulze, 2002). As a dietary supplement produced by a variety of unregulated manufacturers, there have been some concerns regarding product quality. Since creatine is now being used as a therapeutic for disease treatment and is more widely consumed by patients because of its reported potential benefits, apparent safety, and ready availability, additional regulation may be warranted. Overall, however, based on current scientific knowledge, oral creatine supplementation can be regarded as safe (Tarnopolsky & Beal, 2001; Wyss & Schulze, 2002).

6. Conclusion

Neuroprotective compounds targeting identified pathologic mechanisms of disease have the potential to delay the onset and slow the progression of HD. A large number of studies in HD patients and HD model systems have validated energy insufficiency as a promising therapeutic target. Compounds such as creatine and coenzyme Q10 buffer neuronal energy demands and are attractive candidates for targeting this important disease mechanism. Creatine has some advantages, which include lower cost, more straightforward bioavailability, and biomarkers that can be used in vivo. Given the apparent safety and tolerability of creatine, it may be especially well suited for long-term use to delay onset in asymptomatic HD gene-positive individuals. For the same reasons, creatine is also well suited for use in combination with neuroprotective agents targeting other pathologic mechanisms of disease. It is becoming clear that the most effective neuroprotection for HD and other neurological disorders will arise from polytherapies derived from safe and well-tolerated drugs, providing significantly greater efficacy. Preclinical studies testing creatine in combination with other neuroprotective agents are needed to determine which combinations might have the most promise for translation to human clinical trials. A definitive and fully powered phase III clinical trial of creatine, testing its neuroprotective ability, is needed in presymptomatic or symptomatic patients with HD. Ongoing early phase studies will soon determine an optimal dose range for HD patients and provide useful biomarkers.

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Review

Nitrate in vegetables: toxicity, content, intake and EC regulation

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Abstract: Nitrate content is an important quality characteristic of vegetables. Vegetable nitrate content is of interest to governments and regulators owing to the possible implications for health and to check that controls on the content are effective. Nitrate itself is relatively non-toxic but its metabolites may produce a number of health effects. Until recently nitrate was perceived as a purely harmful dietary component which causes infantile methaemoglobinaemia, carcinogenesis and possibly even teratogenesis. Recent research studies suggest that nitrate is actually a key part of our bodies' defences against gastroenteritis. In this review are reported: (1) vegetable classification as a function of nitrate accumulation; (2) vegetable contribution to the total dietary exposure of nitrate; (3) European Commission Regulation No. 563/2002 which sets limits for nitrate in lettuce and spinach; (4) the maximum levels set in some countries for beetroot, cabbage, carrot, celery, endive, Lamb's lettuce, potato, radish and rocket; (5) the results of surveys on the nitrate content of vegetables in Italy and other European countries.

Keywords: nitrate content; nitrate toxicity; vegetable quality; limits for nitrate

INTRODUCTION

Nitrate is a naturally occurring form of nitrogen and is an integral part of the nitrogen cycle in the environment. Nitrate is formed from fertilizers, decaying plants, manure and other organic residues. It is found in the air, soil, water and food (particularly in vegetables) and is produced naturally within the human body.[1–4] It is also used as a food additive, mainly as a preservative and antimicrobial agent.[3,4] It is used in foods such as cheese and cheese products, raw and processed meats, edible casings, processed fish, fish products, spirits and liqueurs.

Due to the increased use of synthetic nitrogen fertilizers and livestock manure in intensive agriculture, vegetables and drinking water may contain higher concentrations of nitrate than in the past.

NITRATE TOXICITY

The presence of nitrate in vegetables, as in water and generally in other foods, is a serious threat to man's health. Nitrate per se is relatively non-toxic,[3,5] but approximately 5% of all ingested nitrate is converted in saliva and the gastrointestinal tract to the more toxic nitrite.[6,7] The only chronic toxic effects of nitrate are those resulting from the nitrite formed by its reduction by bacterial enzymes.[5] Nitrite and *N*-nitroso compounds, which form when nitrite binds to other substances before or after ingestion (for example, the amines derived from proteins), are toxic and can lead to severe pathologies in humans.[8,9] Thus, the assessment of the health risk of nitrate to humans should encompass the toxicity of both nitrite and *N*-nitroso compounds.[8]

Sources of nitrate, nitrite and *N*-nitroso compounds are normally exogenous,[10] but endogenous formation of these compounds has also been demonstrated in both laboratory animals and humans.[2]

The best-known effect of nitrite is its ability to react with haemoglobin (oxyHb) to form methaemoglobin (metHb) and nitrate:

$$NO_2^- + \text{oxyHb}(Fe^{2+}) \longrightarrow \text{metHb}(Fe^{3+}) + NO_3^-$$

As a consequence of the formation of metHb the oxygen delivery to tissue is impaired.[11,5]

Once the proportion of metHb reaches 10% of normal Hb levels, clinical symptoms (from cyanosis—the blue discoloration of the skin due to the presence of deoxygenated blood—through to asphyxia—suffocation) occur. This potentially fatal condition is known as methaemoglobinaemia, or blue baby syndrome.[1,11] Children and adults are far less susceptible to methaemoglobinaemia

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than young infants, because of the induction of methaemoglobin reductase during the physiological post-weaning period. Babies less than three months old are particularly susceptible to methaemoglobinaemia. This is believed to occur because of infants' higher levels of fetal oxyHb still present in the blood, which oxidizes to metHb more readily than non-fetal oxyHb. In addition, infants have less of the reductase needed to reconvert the metHb back to oxyHb, have a proportionately higher intake of nitrate through drinking water by body weight, and have a higher reduction of nitrate to nitrite due to low gastric acidity.[5,12]

Two cases of blue baby syndrome were recently investigated in Wisconsin (USA). Both cases involved infants who became ill after being fed formula that was reconstituted with water from private wells. Water samples collected from these wells during the infants' illnesses contained nitrate N concentrations of 22.9 and 27.4 mg L^{-1}.[11]

Nitrite as such, and nitrate when reduced to nitrite, may react with amines or amides to form carcinogenic *N*-nitroso compounds.[5] Nitrosation can occur mainly in two situations: (1) during storage and ripening of the food product[1] and (2) in the stomach from the action of salivary nitrite produced through enzymatic reduction of endogenous or exogenous nitrate.[13] Since the discovery of the carcinogenicity of *N*-nitrosodimethylamine in rats by Magee and Barnes,[14] *N*-nitroso compounds have been shown to be carcinogenic in more than 40 animal species.[2] These include mammals, birds, reptiles and fish, and there is no reason to suspect that human beings are uniquely resistant.[15]

Several authors have suggested that the risk for the development of stomach cancer is positively correlated with three factors: (1) the nitrate level of drinking water, (2) the urinary excretion of nitrate and (3) the occurrence of atrophic gastritis.[8] Epidemiological studies have not provided any evidence that there is an increased risk of cancer related to high nitrate intake from sources other than vegetables.[2] In some cases studies revealed a negative correlation between nitrate intake and gastric cancer,[2,16,17] because vegetables are an excellent source of vitamins, minerals and biologically active compounds.[18,19]

Some years ago, Vermeer *et al.*[20] showed the endogenous formation of carcinogenic *N*-nitroso compounds (*N*-nitrosodimethylamine and *N*-nitrosopiperidine) after intake of nitrate at the acceptable daily intake (ADI, see below) level in combination with a fish meal rich in amines as nitrosatable precursors. The vegetables used in the research (cauliflower, peas, carrots and green beans) were low in nitrate, and their mean vitamin C content (an anti-carcinogenic agent) was approximately 170 mg kg^{-1} vegetables. Thus, the amount of vitamin C (and other antioxidants) in these vegetables appeared insufficient to prevent nitrosamine formation.[20] The same Danish research group has shown that nitrate can interfere with iodine uptake by the thyroid, resulting in hypertrophy of the thyroid, the gland responsible for many of the body's endocrine and hormonal functions.[21]

More recently, another research group has shown a positive relationship between the incidence of childhood-onset insulin-dependent diabetes mellitus and levels of nitrate in drinking water.[22] Their findings suggest that the threshold for the effect is 15 mg L^{-1} of NO_3 (less than one-third of the EC limit for nitrate in drinking water), which is considered both worrying[23] and puzzling.[24]

Recent research suggests that dietary nitrate may have beneficial effects, based on the hypothesis that nitric oxide (NO) formed in the stomach from dietary nitrate has antimicrobial effects on gut pathogens and a role in host defence.[12,25,26]

The potential beneficial effects of nitrate have been the subject of limited research; however, there was enough evidence in several areas including prevention of microbial infections, reduction of hypertension and cardiovascular diseases, and reduction in the risk of gastric cancer, to lead two researchers to publish a paper entitled 'Are you taking your nitrate?'[24]

Finally, it is important to cite the text 'Nitrate and man: toxic, harmless or beneficial?' which, as suggested by the title, is a broad-ranging review of the role of nitrate in human health.[27] The authors critically review the evidence relating to the reported adverse effects of nitrate and note that a plausible hypothesis (the toxicity of nitrate) has been transformed into a practically sacrosanct dogma, in spite of the lack of proof.[27]

However, an intake of vegetables and consumption of drinking water with such a high nitrate content that the ADI is exceeded for a prolonged period should be avoided.[28] Thus, in order to gain as much as possible from the indisputable benefits of vegetables, a reduction in nitrate levels is highly desirable for consumers and probably profitable for farmers.[29]

ACCEPTABLE DAILY INTAKE

The concept of ADI is defined by the Joint Expert Committee of the Food and Agriculture (JECFA) Organization of the United Nations/World Health Organization (WHO) for substances intentionally added to food or for contaminants (pesticides, herbicides and fertilizers).[3,4] In view of the well-known benefits of vegetables and the lack of data on the possible effects of vegetable matrices on the bioavailability of nitrate, JECFA considered it to be inappropriate to compare exposure to nitrate from vegetables with ADI or to derive limits for nitrate in vegetables directly from it.[3] In the absence of an appropriate alternative approach in the literature the consequences of nitrate intake exceeding the ADI are discussed.

The JECFA and the European Commission's Scientific Committee on Food (SCF) have set an ADI for NO_3 of 0–3.7 mg kg^{-1} bodyweight.[3,4,30–32]

Table 1. Estimated intakes of NO_3 from sources other than food additives at the global level (after Hambridge[34])

Regional diet	Intake (mg day^{-1})	ADI[a] (μg mg^{-1})	Major contributors to total intake (μg mg^{-1})			
			Vegetables	Water	Cereals	Fruit
Middle Eastern	40	200	650	200	100	50
Far Eastern	28	100	450	300	150	100
African	20	100	300	400	150	100
Latin American	55	250	650	150	50	100
European	155	700	900	50	<50	50

[a] Based on 60 kg body weight.

The USA Environmental Protection Agency (EPA) Reference Dose (RfD) for nitrate is 1.6 mg nitrate nitrogen kg^{-1} bodyweight (bw) per day (equivalent to about 7.0 mg NO_3 kg^{-1} bw per day).[5]

The JECFA and SCF have proposed an ADI for NO_2 of 0–0.07[8,9] and 0–0.06 mg NO_2 kg^{-1} bw[32], respectively, while the EPA has set an RfD of 0.1 mg nitrite nitrogen kg^{-1} bw per day (equivalent to 0.33 mg NO_2 kg^{-1} bw per day).[5]

The SCF retains that the ADIs are applicable to all sources of dietary exposure.[30,31]

NITRATE INTAKE

The three main sources of nitrate intakes are vegetables, water and cured meat.[1,33]

Vegetables constitute the major dietary source of nitrate, generally providing from 300 to 940 mg g^{-1} of the daily dietary intake (Tables 1 and 2). Their contribution to nitrite intake is low, and in fact lower than that from cured meat products.[1,10,33] Nitrite is found in plant foodstuffs, typically 1–2 mg kg^{-1} of fresh vegetable weight.[46] Potatoes, however, can contain up to 60 mg NO_2 kg^{-1}.[46] Higher amounts of nitrite are found in contaminated food or in broken vegetable tissues in food stored for several days at room temperature.[33]

Table 2. Estimate of nitrate daily intake (drinking water not included) and contribution of vegetables in various countries

Country	NO_3 (mg person^{-1})	Vegetable contribution[c] (μg mg^{-1})	Reference
Belgium	148[a]	93	Dejonckheere *et al.*[35]
European Union	18–131[b]	100	SCF[32]
Finland	77	92	Dich *et al.*[36]
France	121	85	Cornée *et al.*[37]
Germany	68	72	Selenka and Brand-Grimm[38]
Italy	149	90	CSS[39]
Poland	65–85	ND	Borawska *et al.*[40]
Spain	60	ND	ACAPV[41]
Sweden	50	ND	Jagerstad and Nilsson[42]
The Netherlands	52	ND	Ellen *et al.*[43]
UK	95	94	Knight *et al.*[44]
USA	73	90	NRC[45]

[a] Included only vegetables and fruit.
[b] Included only vegetables.
[c] Not detected.

Compared with the current ADIs, the ingestion of only 100 g of raw vegetables with a nitrate concentration of 2500 mg kg^{-1} will already lead to an intake of 250 mg NO_3. Consuming this item alone, for a person of 60 kg, would exceed the ADI for nitrate by 13%. Calculating in the partial conversion of nitrate to nitrite (5%) after such consumption, the current SCF ADI for nitrite (0.06 mg kg^{-1} bw) would be exceeded by 247%.

A statistical exposure model has shown that in the adult population in the Netherlands 15% had daily intakes regularly exceeding the ADI;[47] in young children this may rise to 45%.[47]

In its last report, the SCF[32] reviewed from a public health standpoint the presence of nitrate in foodstuffs in general, and vegetables in particular, and stated that the total intake of nitrate is normally well below the acceptable daily intake (Table 2). The major sources are potatoes and lettuce, the first because they are vegetables consumed in the largest quantity mainly (Table 3), the latter due to its high nitrate content.[32] The SCF[31,32] recommended continuation of efforts to reduce exposure to nitrate via food and water since nitrate can be converted into nitrite and nitrosamines, and urged that good agricultural practices are adopted to ensure nitrate levels are as low as reasonably achievable.

In previous research, nitrate intake from vegetables was estimated using the data on average daily per capita consumption of vegetables provided by the Italian National Institute of Nutrition[49] and on average nitrate content from the research work: nitrate daily intake from vegetables was 71 mg d^{-1}; over 300 mg g^{-1} of nitrate intake was derived from the consumption of lettuce and Swiss chard.[50]

Dietary exposures to nitrate for vegetarians are very similar to those of other consumers and are below the ADI. The average dietary exposure of the vegetarians in a UK study was 83 mg d^{-1} and the highest nitrate exposure was 209 mg d^{-1}.[51,52]

NITRATE CONTENT IN VEGETABLES

There are several factors affecting NO_3 uptake and accumulation in vegetable tissues, e.g. genetic

Table 3. Food consumption and nitrate daily intake in some countries of European Union (after Schuddeboom[48])

Food group	Country	Food consumption (g person^{-1})	Average concentration (mg kg^{-1} fm)	NO_3 intake (mg person^{-1})	Contribution to nitrate intake (μg mg^{-1})
Potato	Germany	112	93	10.5	113
	Denmark	166	80	13.3	185
	The Netherlands	131	60	7.9	66
	UK	160	120	19.0	339
Other vegetables	Germany	73	721	52.6	564
	Denmark	114	440	50.2	697
	The Netherlands	150	800	120.0	857
	UK	162	136	22.0	393
Fruit	Germany	101	70	7.1	76
	Denmark	120	30	3.6	50
	The Netherlands	125	20	2.5	18
	UK	91	25	2.3	41

Table 4. Classification of vegetables according to NO_3 content (mg kg^{-1} fm)

Very low (<200)	Low (200–500)	Middle (500–1000)	High (1000–2500)	Very high (>2500)
Artichoke	Broccoli	Cabbage	Celeriac	Celery
Asparagus	Carrot	'Cima di rapa' (broccoli rab)	Chinese cabbage	Chervil
Broad bean	Cauliflower	Dill	Endive	Cress
Brussels sprouts Eggplant	Cucumber	'Radicchio'	Escarola	Lamb's lettuce
Garlic	Pumpkin	Savoy cabbage	Fennel	Lettuce
Onion	'Puntarelle' chicory	Turnip	Kohlrabi	Radish
Green bean			Leaf chicory	Red beetroot
Melon			Leek	Rocket
Mushroom			Parsley	Spinach
Pea				Swiss chard
Pepper				
Potato				
Summer squash				
Sweet potato				
Tomato				
Watermelon				

factors, environmental factors (atmospheric humidity, substrate water content, temperature, irradiance, photoperiod) and agricultural factors (nitrogen doses and chemical forms, availability of other nutrients, use of herbicides, etc.).[53–55]

Of the factors studied, nitrogen fertilization and light intensity have been identified as the major factors that influence nitrate content in vegetables.[56] In particular, light intensity and nitrate content in soil before or at harvest are known to be critical factors in determining nitrate levels in spinach[53] or other leafy vegetables.[55,57]

Generally, nitrate-accumulating vegetables belong to the families of Brassicaceae (rocket, radish, mustard), Chenopodiaceae (beetroot, Swiss chard, spinach) and Amarantaceae; but also Asteraceae (lettuce) and Apiaceae (celery, parsley) include species with high nitrate contents (Table 4).

Nitrate content can vary also within species, cultivars and even genotypes with different ploidy.[58]

The differing capacities to accumulate nitrate can be correlated with differing location of the nitrate reductase activity,[59–61] as well as to differing degree of nitrate absorption and transfer in the plant.[53,62]

Nitrate content differs in the various parts of a plant.[50] Indeed, the vegetable organs can be listed by decreasing nitrate content as follows: petiole > leaf > stem > root > inflorescence > tuber > bulb > fruit > seed.[50,63]

The highest nitrate-accumulating vegetable is rocket,[50] a leafy vegetable popular in the Mediterranean region.[55,64,65] A number of species of the Brassicaceae family are grouped under the name of rocket belonging to the *Eruca* Miller and *Diplotaxis* DC. genera. *Eruca* is present in both wild and cultivated forms; *Diplotaxis* is known as a wild type. In *Diplotaxis*, two surveys carried out in Italy show NO_3 content reaches up to 9300 mg kg^{-1}.[50,66]

Rocket absorbs NO_3 very quickly[64,65] and NO_3 concentration in leaves can be much higher than in the growth medium. With 1 mmol L^{-1} NO_3 nitrogen in a hydroponic nutrient solution, NO_3 accumulation ratio (expressed as the ratio between the concentration in leaves and in nutrient solution) was, respectively, 55 for *E. vesicaria* and 101 for *D. tenuifolia*.[67]

Soil-less systems offer a clear advantage to traditional ones in the management and control of plant

mineral uptake during the various phases of the growing cycle. One advantage of these growing systems is that they can be used to produce vegetables with low nitrate accumulation.[55,65,68]

LIMITS TO MAXIMUM LEVELS OF NITRATE IN VEGETABLES

To protect public health in response to the SCF's considerations of nitrate in food,[30–32] in 1997 the European Member States agreed an EC Regulation setting limits for nitrate in lettuce and spinach (EC Regulation No. 194/97).[69] The main purpose of this EC Regulation was to harmonize limits for nitrate in these vegetables, as the different national limits set by some Member States were causing trade difficulties across the European Union.

On 2 April 2002 the European Commission amended EC Regulation No. 194/97 (already amended with some periphrasis from EC Regulation No. 864/1999[70] and 466/2001[71]) and adopted EC Regulation No. 563/2002.[72] The maximum levels set by this Regulation are summarized in Table 5. The limits vary according to season, with higher nitrate levels permitted in crops grown in winter compared with those grown in the summer. Lower limits are fixed for open-grown lettuce than for lettuce grown under glass, and in order to allow effective control the limits set for open-grown lettuce should apply also to lettuce grown under glass in the absence of precise labelling. Lower limits are fixed for 'iceberg' than other types of lettuce.

The differences between nitrate levels in different varieties have been most extensively studied in relation to lettuce where open leaf varieties generally have higher nitrate concentrations than tight-headed varieties such as iceberg.[32]

EC Regulation No. 563/2002 states that 'in some regions nitrate levels are reported to be frequently higher than those set in the Annex of Regulation (EC) No 466/2001, although the general trend shows that the levels of nitrate in lettuce are decreasing'.[72] The levels of nitrate in spinach show no clear trend for reduction. So, 'some Member States need to maintain the established transitional period to authorise the placing on the home market of lettuce and/or spinach grown and intended for consumption in their territory'.[72] This derogation requires annual monitoring to be carried out to demonstrate that nitrate levels in these crops are acceptable on public health grounds and that growers follow a 'code of good agricultural practice'.[72]

The UK is currently applying this derogation for both lettuce and spinach along with Ireland. A derogation for spinach presently applies in Finland, Denmark and the Netherlands. However, maximum limits do apply to lettuce and spinach imported from Member States and third countries. The derogation for lettuce ended on 1 January 2005. An extension of this derogation is currently under consideration within the EU. The derogation for spinach is currently being reviewed.[72]

Nevertheless, no official method has been published in EU legislation and nitrate levels in vegetables are generally assayed by modifying the protocols used for other foods.

Limits to maximum levels of nitrate for trade in other vegetables are set in some European countries (Table 6). For potato, several countries have put forward the proposal of 'guidelines' for nitrate content (in Germany, for instance, only tubers with less than 200 mg kg^{-1} fresh matter (fm) are accepted), while in Poland there is a maximum limit of 183 mg kg^{-1} fm.[73]

Rocket and other Italian export vegetable (e.g. potato) sales contracts include very strict clauses, for instance with Switzerland and Germany. Namely, nitrate content for rocket is required not to exceed 2.5–4.0 g kg^{-1} fm, which is a very strict threshold that is difficult to respect on account of the high accumulation of nitrate in rocket, even when reduced amounts of nitrate are used in its cultivation.[74]

No nitrate standards for vegetables have been introduced in the USA. In China, a suggested maximum level of nitrate in vegetables of 3100 mg kg^{-1} has been established.[75]

Table 5. Maximum levels (limits) for the nitrate (mg kg^{-1} fm) in lettuce and spinach according to European Commission Regulation (EC) No. 563/2002[72]

Product	Harvest period	NO_3
Fresh spinach[a] (*Spinacia oleracea* L.)	Harvested 1 November to 31 March	3000
	Harvested 1 April to 31 October	2500
Preserved, deep-frozen or frozen spinach		2000
Fresh lettuce (*Lactuca sativa* L.) (protected and open-grown lettuce) excluding 'iceberg' type	Harvested 1 October to 31 March:	
	— lettuce grown under cover	4500
	— grown in the open air	4000
	Harvested 1 April to 30 September:	3500
	— lettuce grown under cover	2500
	— lettuce grown in the open air	
'Iceberg' type lettuces	Lettuce grown under cover	2500
	Lettuce grown in the open air	2000

[a] The maximum levels for fresh spinach do not apply for fresh spinach subject to processing and which is directly transported in bulk from field to processing plant.

Table 6. Maximum levels (limits) of NO_3 (mg kg^{-1} fm) to trade various vegetables in some European countries (after Santamaria[33])

Vegetable	Austria	Belgium	Germany	Netherlands	Switzerland
Carrot	1500				
Red beetroot	4500		3000	3500	3500
Endive (summer)	2500	2000		2500	2500
Indivia (winter)	3500	2000		3500	2500
Cabbage	1500				
Radish					3500
Celery (green)		5000			
Celery (white)		4000			
Lamb's lettuce		3500	2500		

On 7 April 2004, the European Commission established the maximum permitted level for nitrate in baby foods and processed cereal-based foods for infants and young children of 200 mg kg^{-1} on an 'as sold' basis.[76]

CONCLUSIONS

Although current epidemiological data provide conflicting evidence regarding the potential long-term health risks of nitrate levels encountered in the diet, it is widely accepted that the reduction of dietary nitrate is a desirable preventive measure. The maximum allowable nitrate levels in vegetables should not exceed levels that reflect good agricultural practices.

A reduction in nitrate content can, however, represent added value for vegetable products (already rich in carotenoids, vitamins C and E, selenium, dietary fibre, plant sterols, glucosinolates and indoles, isothiocyanates, flavonoids, phenols, etc.).

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CHRONIC SUPPLEMENTATION OF CREATINE AND VITAMINS C AND E INCREASES SURVIVAL AND IMPROVES BIOCHEMICAL PARAMETERS AFTER DOXORUBICIN TREATMENT IN RATS

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SUMMARY

1. Doxorubicin is an anti-cancer drug with well-described effects against a wide range of tumours. However, doxorubicin also exhibits dose-dependent cytotoxicity. The purpose of the present study was to determine whether chronic supplementation of creatine or a mix of vitamins C and E could increase survival and improve plasma parameters 48 h after doxorubicin treatment.

2. Rats were divided into four groups: (i) saline (control); (ii) doxorubicin treated; (iii) a creatine (0.2 g/kg per day)-supplemented group; and (iv) a vitamin C (250 mg/kg per day) and E (400 IU/kg per day)-supplemented group. After 30 days supplementation of rats with either creatine or the vitamins, one dose of doxorubicin (15 mg/kg, i.p.) was administered.

3. There was no difference in weight loss among the groups until the 3rd day after doxorubicin treatment, but the creatine- and vitamin-supplemented groups lived longer compared with the doxorubicin only treated group (6, 7 and 3 days, respectively). The doxorubicin-treated group lost 13.4% bodyweight over 3 days, whereas the creatine- and vitamin-supplemented groups lost approximately 35% 3 days after the administration of doxorubicin. Doxorubicin treatment resulted in an increase in alanine aminotransferase (ALT; $P < 0.05$), lactate dehydrogenase (LDH; $P < 0.05$), urea ($P < 0.05$) and creatinine ($P < 0.05$) compared with levels observed in the control group. Conversely, creatine supplementation promoted a partial return to control values for LDH ($P < 0.05$) and creatinine ($P < 0.05$), whereas the vitamin mix reversed the changes in ALT ($P < 0.05$), LDH ($P < 0.05$), urea ($P < 0.05$) and creatinine ($P < 0.05$).

4. In conclusion, the results of the present study indicate that the two supplementation protocols decreased the cytotoxic effects of doxorubicin and that a protective effect was more noticeable in animals supplemented with the mixture of vitamins C and E.

Key words: adriamycin, cellular damage, creatine, injury, vitamin C, vitamin E.

INTRODUCTION

Doxorubicin is a potent chemotherapeutic drug from the anthracycline family. Doxorubicin was discovered in the early 1960s and represented a considerable advance in the fight against cancer.[1–5] However, treatment of cancer patients with doxorubicin has several acute and chronic side-effects. The acute effects are mainly myelosuppression, nausea, vomiting, weight loss, arrhythmias and decreased survival, whereas the main chronic effect of doxorubicin is severe cardiomyopathy with congestive heart failure.[1,5–10]

Tissues such as the kidneys, brain, liver and skeletal muscle are affected by doxorubicin.[10–14] Doxorubicin-induced cytotoxicity is mediated through different mechanisms, including membrane lipid peroxidation,[15] free radical formation,[16] mitochondrial damage[17] and iron-dependent oxidative damage to biological macromolecules.[18] Pharmacological methods to interrupt the cycle of reactive oxygen species (ROS) generation include several anti-oxidant substances, such as glutamine,[8] melatonin[7,8] and L-carnitine. Unfortunately, none of these compounds has been proven to be cardioprotective in patients receiving doxorubicin.

Several studies have evaluated the isolated effects of supplementation with vitamins C[6] or E[3,19–21] on cytotoxicity induced by doxorubicin. These studies found positive effects of these treatments. Vitamin C (ascorbic acid) is an essential micronutrient required for normal metabolic function. Two major properties of vitamin C make it an ideal anti-oxidant. The first is the low one-electron reduction potential of both ascorbate and its one-electron oxidation product, the ascorbyl-radical, which is derived from the ene-dial functional group in the molecule. The second major property that makes vitamin C such an effective anti-oxidant is the stability and low reactivity of the ascorbyl radical formed when ascorbate scavengers a reactive oxygen or nitrogen species.[22] Oral supplementation with vitamin C has also been shown to reduce lipid peroxidation and other markers of doxorubicin toxicity.[6]

Vitamin E (α-tocopherol) has several biological functions (e.g. metabolic functions, inhibition of cancer initiation and stimulation of the immune response) and oral supplementation with vitamin E has been shown to protect cells and tissues from oxidative damage (mainly from lipid peroxidation) induced by doxorubicin.[19]

Creatine has anti-oxidant capacity[23] and may have an important role in rebuilding heart structure and function recovery after heart failure.[23] In athletes, creatine can promote an increase in bodyweight, improve performance (mainly in anaerobic sports), enhance glycogen supercompensation and glycogen stores and stimulate protein synthesis.[24–27] Increases in cellular creatine content as creatine phosphate

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can improve mitochondrial creatine kinase activity and affect energy production by changing the rate of ATP resynthesis[27,28] and preventing failure of ATP resynthesis owing to its anti-oxidant capacity; thus, creatine supplementation may also be important in protecting against doxorubicin-induced cytotoxicity.

Based on these observations, the aim of the present study was to determine whether chronic supplementation with creatine or a mix of vitamins C and E is able to promote a change in survival, weight loss and biochemistry parameters in rats after doxorubicin treatment.

METHODS

Animals

Male Wistar rats,weighing 150–200 g at the beginning of the experiment, were obtained from the Biomedical Science Institute animal house, University of São Paulo, Brazil. Rats were kept under a constant 12 h light–dark cycle (lights on at 0700 hours) at 22 ± 2°C and 60 ± 5% humidity. Rats were kept in collective cages (five animals per cage) and received water and food *ad libitum*. Rats were randomly divided into four groups: (i) saline control (n = 12); (ii) doxorubicin treated (n = 15); (ii) creatine + doxorubicin treated (n = 15); and (iv) vitamins C and E + doxorubicin treatment (n = 15). Creatine was administered at 0.2 g/kg per day for 30 days and vitamins C and E were administered at 0.25 mg/kg per day and 400 IU/kg per day, respectively, for 30 days by gavage. After 30 days supplementation, the doxorubicin-treated and creatine- and vitamin-supplemented groups received 15 mg/kg, i.p., doxorubicin cloridrate (Eurofarma Laboratory, Campinas, Brazil); control animals received an equal volume of saline. Creatine and vitamin supplementation was continued throughout the doxorubicin administration and animals were killed by decapitation either 48 h after injection of doxorubicin or, in another set of experiments, were kept alive to evaluate animal survival after doxorubicin treatment. Whole blood was drawn into heparinized before being centrifuged at 3000 *g* for 15 min at 4°C. Plasma was removed and kept frozen at –80°C for later determination.

Bodyweight

All animals were weighed weekly during the experiment and daily after doxorubicin treatment between 0700 and 0800 hours using an Ohaus balance (Marte, São Paulo, Brazil).

Biochemical parameters

Lactate dehydrogenase (LDH) was assayed enzymatically in assay medium containing Tris-HCl (50 mmol/L), NADH (0.17 mmol/L), sodium pyruvate (0.3 mmol/L) and KCN (1 mmol/L). The final pH was 7.3 and the assay was started by the addition of pyruvate as described by Zammit and Newsholme.[29]

Creatinine was assayed using a colourimetric method in medium containing picric acid (60 mmol/L), NaOH (110 mmol/L), sodium carbonate (75 mmol/L) and acetic acid (12 mmol/L) with a commercially available kit (Bioclin, Belo Horizonte, Brazil).

Urea was assayed enzymatically in medium containing phosphate buffer (100 mmol/L), pH 7.5, sodium nitroprussate (10 mmol/L), sodium salicylate (60 mmol/L), NaOH (1.5 mol/L), sodium hyplochloride (10 mmol/L) and urease (10 000 U/L) using a commercially available kit (Bioclin).

Alanine aminotransferase (ALT) was assayed enzymatically in medium containing NADH (0.18 mmol/L), lactate dehydrogenase (1200 U/L), α-ketoglutarate (15 mmol/L), Tris-buffer (100 mmol/L), pH 7.8, alanine (500 mmol/L) and sodium azide (15 mmol/L) using a commercially available kit (Bioclin).

All data are presented as the mean±SEM. Data were evaluated using SigmaStat 3.1 for statistical program. Statistical differences were determined using analysis of variance with Bonferroni post hoc comparison. $P < 0.05$ was considered statistically significant.

Table 1 Effects supplementation with creatine or vitamins C and E on deaths after doxorubicin treatment

	No. rats dying after doxorubicin treatment						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Doxorubicin	–	6	4	–	–	–	–
Creatine	–	–	–	2	1	7	–
Vitamins C + E	–	–	–	2	1	4	3

Table 2 Effect of doxorubicin treatment and supplementation with creatine or vitamins C and E on bodyweight, bodyweight changes and bodyweight wasting

Groups	Bodyweight	Bodyweight change (g)		Bodyweight wasting (%)	
	Day 0	Day 3	Day 6	Day 3	Day 6
Doxorubicin	252.8 ± 31	221.0 ± 0	–	12.6	–
Creatine	256.8 ± 17	224.6 ± 15	176.3 ± 8*	12.4	31.6
Vitamins C + E	264.0 ± 13	229.2 ± 9	194.3 ± 5*†	13.2	26.5

Data are the mean±SD (n = 10 in each group). *$P < 0.05$ compared with Day 0; †$P < 0.05$ compared with creatine supplementation.

RESULTS

The effect of supplementation and doxorubicin treatment on survival was determined by observing survival after doxorubicin treatment of 30 rats, as indicated in Table 1. There were no deaths on the first day after treatment. On the second day, six animals from the doxorubicin group died (60%) and, on the 3rd day, the other four animals in this group died. All animals in the doxorubicin-treated group had died by 3 days.

Conversely, animals in the supplemented groups had longer survival compared with the doxorubicin-treated group. On the fourth day, one animal from each of the creatine- and vitamin-supplemented groups died. On the 5th day, again two animals from each of the creatine- and vitamin-supplemented groups died. However, on the 6th day there was a considerable difference in survival between the creatine- and vitamin-supplemented groups, because seven animals in the former group died compared with only four in the latter group. Finally, on the 7th day, three animals from the vitamin-supplemented group died (Table 1).

Bodyweight wasting is given in Table 2. Animals in the doxorubicin-treated group lost 13% of bodyweight 3 days after doxorubicin treatment (weight 253 ± 31 and 221 ± 0 g on Days 0 and 3, respectively). Animals in the creatine-supplemented group lost 12.4% of bodyweight 3 days after doxorubicin treatment (257 ± 17 g and 225 ± 15 g on Days 0 and 3, respectively). In this group, on the 4th, 5th and 6th days after doxorubicin treatment, there were decreases in bodyweight of 9.7, 4.8 and 8.8%, respectively, resulting in a significant difference in bodyweight between Day 0 and Day 6 (257 ± 17 *vs* 176 ± 8 g, respectively; $P < 0.05$). Rats treated with the vitamin mix showed a similar profile of bodyweight loss over the first 3 days after doxorubicin (13.2%; 264 ± 13 and 229 ± 9 g on

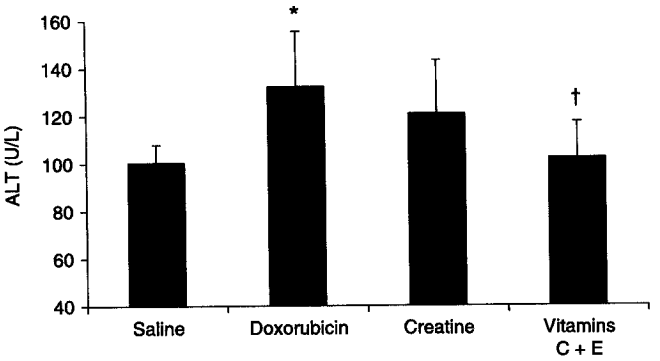

Fig. 1 Effects of supplementation with creatine or vitamins C and E on plasma alanine aminotransferase (ALT) activity after treatment with doxorubicin compared with saline control and doxorubicin alone. Data are the mean±SD ($n = 12$–15). *$P < 0.05$ compared with saline; †$P < 0.05$ compared with doxorubicin alone.

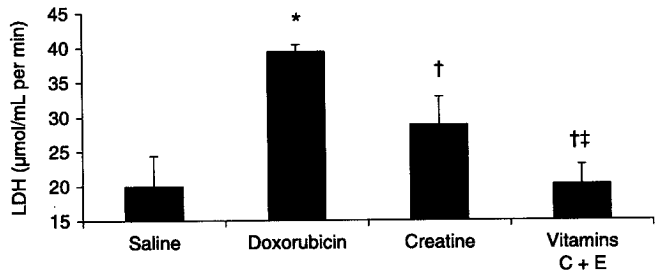

Fig. 2 Effects of supplementation with creatine or vitamins C and E on plasma lactate dehydrogenase (LDH) activity after treatment with doxorubicin compared with saline control and doxorubicin alone. Data are the mean±SD ($n = 12$–15). *$P < 0.05$ compared with saline; †$P < 0.05$ compared with doxorubicin alone; ‡$P < 0.05$ compared with creatine supplementation.

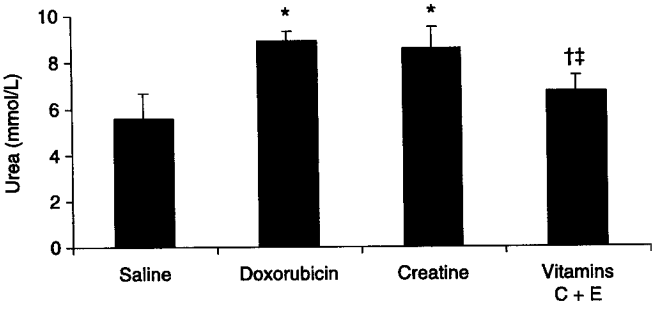

Fig. 3 Effects of supplementation with creatine or vitamins C and E on urea after treatment with doxorubicin compared with saline control and doxorubicin alone. Data are the mean±SD ($n = 12$–15). *$P < 0.05$ compared with saline; †$P < 0.05$ compared with doxorubicin alone; ‡$P < 0.05$ compared with creatine supplementation.

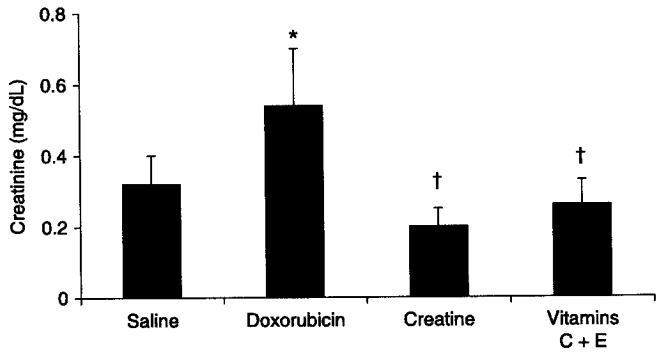

Fig. 4 Effect of supplementation with creatine or vitamins C and E on creatinine after treatment with doxorubicin compared with saline control and doxorubicin alone. Data are the mean±SD ($n = 12$–15). *$P < 0.05$ compared with saline; †$P < 0.05$ compared with doxorubicin alone.

Days 0 and 3, respectively) and, on the 4th, 5th, 6th and 7th days after doxorubicin treatment, bodyweight decreased by 5.4, 5.2, 4.5 and 5.1%, respectively, resulting in a significant difference between Day 0 and Day 6 (264 ± 13 *vs* 194 ± 5 g; $P < 0.05$). On the 6th day after doxorubicin treatment, there was a significant difference in bodyweight between the creatine- and vitamin-supplemented (176 ± 8 *vs* 194 ± 5 g, respectively; $P < 0.05$), with the vitamin-supplemented group losing less weight and having 1 more day of survival.

Table 2 shows the percentage of bodyweight wasting accumulated over the 7 days after doxorubicin administration. On the 6th day, accumulated bodyweight wasting was 31.6% in the creatine-supplemented group, whereas in the vitamin-supplemented group the value was 26.5%.

The administration of doxorubicin induced a significant increase (32%) in plasma ALT activity compared with the sailne-treated group (132.3 ± 23.1 and 100.3 ± 7.6 U/L, respectively; $P < 0.05$). Supplementation with the vitamins C and E mix was effective in reducing ALT plasma activity (102.2 ± 15.2 U/L; 29% reduction) compared with the doxorubicin-treated group ($P < 0.05$; Fig. 1).

Similarly, plasma LDH activity was approximately 97.6% higher in the doxorubicin-treated group compared with the saline-treated group (39.4 ± 1.0 and 19.9 ± 4.4 μmol/mL per min, respectively; $P < 0.05$), whereas plasma LDH activity was lower in the creatine-supplemented compared with the doxorubicin-treated group, although LDH activity remained higher than in the saline-treated group. Conversely, in the vitamin-supplemented group, LDH activity was similar to that in the saline-treated group and was smaller than that in the doxorubicin-treated and creatine-supplemented groups (Fig. 2).

Urea levels were significantly higher ($P < 0.05$) in both the doxorubicin-treated and creatine-supplemented groups (8.96 ± 0.47 and 8.60 ± 0.92 mmol/L, respectively) compared with the saline control group (5.65 ± 1.08 mmol/L). Urea levels in the vitamin-supplemented group (6.73 ± 0.68 mmol/L) were significant lower than those in the doxorubicin-treated and creatine-supplemented groups ($P < 0.05$; Fig. 3).

Figure 4 shows a significance increase in creatinine levels of approximately 68.8% in doxorubicin-treated rats compared with the saline control group (0.54 ± 0.16 and 0.32 ± 0.08 mg/dL, respectively; $P < 0.05$). In the creatine- and vitamin-supplemented groups, creatinine levels were decreased compared with the doxorubicin-treated group (0.20 ± 0.05, 0.26 ± 0.07 and 0.54 ± 0.16 mg/dL, respectively; $P < 0.05$). There were no statistically significant differences between the supplemented and saline-treated groups.

DISCUSSION

Doxorubicin is considered one of the most potent antineoplastic antibiotics, with a broad spectrum of therapeutic activity in the treatment of haematogenous malignancies as well as solid tumours of the lung, breast, thyroid and ovary.[1,10] Unfortunately, the use of this anticancer drug is limited by elevated oxidative cytotoxicity.[1,6,7,10–12,30,31]

In order to reduce doxorubicin cytotoxicity, several anti-oxidant substances have been investigated in experimental models.[32] The present study is the first to investigate the effects of chronic supplementation of creatine or a mix of vitamins C and E on survival, weight loss and biochemical parameters as markers of cellular damage after a doxorubicin dose of 15 mg/kg.

In the present study, a single dose of 15 mg/kg, i.p., doxorubicin was used, despite the fact that 10 mg/kg is well documented to induce cellular damage in several tissues, such as the heart and kidney.[4,10,33,34] In the present study, doxorubicin induced severe body wasting and an average survival period of 3 days after treatment. These changes are concomitant with a significant increase in markers of cellular damage, such as plasma LDH and ALT activity and biochemical parameters (plasma creatinine and urea concentrations).

Others have reported similar results. Oz *et al.*[34] found a fourfold increase in plasma LDH activity 48 h after administration of only one dose of 45 mg/kg doxorubicin, whereas Oz and Ilhan,[35] after reviewing the literature, found an increase of approximately 40% in LDH activity. The present study showed an increase of 97% in LDH activity in the doxorubicin-treated group compared with the saline control group, confirming previous results. In addition, we found a significant 32% increase in plasma ALT activity in the doxorubicin-treated group.

These enzymes are found in large quantities in cardiac and skeletal muscle and are therefore important when detecting accentuated cytotoxicity, with an increased index of cellular damage mainly in the cardiac muscle. Thus, the present results showed that the dose of doxorubicin used was sufficient to induce disruption of cells.

Although there is a substantial amount of literature on doxorubicin-induced cardiotoxicity, there is a general paucity of information on its effects on skeletal muscle. However, there is some evidence for doxorubicin myotoxicity in skeletal muscle. Injection of doxorubicin in rats induces changes in perinucleolar chromatin and segregation of the nucleolus of skeletal muscle.[36] These changes are rapid and occur within 1 h of doxorubicin administration, and it has been reported that the ultrastructural abnormalities are similar to those occurring in cardiac muscle.[36] *In vitro*, doxorubicin suppresses skeletal muscle expression of the transcription factors MyoD and myogenin.[37] Thus, it seems that doxorubicin may impart a degree of myotoxicity, which is further supported by histological studies,[38] In addition, although we have not evaluated cardiac and skeletal muscle mass under any experimental conditions, we would point out that considerable body wasting and an increase in plasma urea concentrations suggest a high rate of proteolysis induced by doxorubicin treatment.

The exact mechanism of the cytotoxic action of doxorubicin is not clear. However, it is widely accepted that oxidative stress and the production of free radicals are important in this process because it has been shown that doxorubicin treatment leads to direct oxidative injury of DNA and generates lipid peroxidation, with a consequent significant cytotoxicity mainly in cardiac muscle.

In the present study, we investigated the ability of two protocols using known anti-oxidants to decrease the harmful effects of doxorubicin. Creatine exhibited excellent anti-oxidant capacity in addition to its anabolic action,[23] whereas isolated vitamins C and E have been tested early, during and after doxorubicin treatment,[1,3,6,19–21] but never as a mixture of vitamins.

The results of the present study indicate that the two supplementation protocols investigated herein achieved the objective of preventing the cytotoxic effects induced by doxorubicin, because both supplemented groups exhibited a reduction in the indices of cellular damage. Although, the creatine-supplemented group showed decreased LDH but not ALT activity after doxorubicin treatment, the group supplemented with the mix of vitamins showed small LDH and ALT activity, suggesting a more effective protective action of that solution. This confirms previous studies that have observed the protective effects of the administration of vitamin C or E against the cytotoxic action of doxorubicin in humans and rodents.[3,6,19–21]

Interestingly, we found an increase of survival in both the creatine- and vitamin-supplemented groups, with differences in bodyweight wasting on the 6th day and 1 more day of survival in the vitamin-supplemented group. Supplementation with the mixture of vitamins C and E was more effective in increasing the animals' survival compared with creatine supplementation and we speculate that the small percentage change in bodyweight during the later days may be less aggressive and cause less damage to the animal, because 30% of animals from vitamin-supplemented group died on the 7th day. The decrease in bodyweight wasting can be attributed to a decrease in cellular damage or simply inhibition of protein synthesis[31] and/or increased proteolysis due to doxorubicin treatment.

A recent study has investigated the beneficial effects of vitamin E (250 mg/kg per day for 15 days) in Ehrlich carcinoma-bearing mice on cardiotoxicity induced by doxorubicin (administration of 4 mg/kg per week, i.p., for 2 weeks).[19] That study found an increase in total protein in the heart, increased glutathione and superoxide dismutase activity and a decrease in malondialdehyde in the vitamin E-supplemented group compared with the doxorubicin-treated group. When a dose of 250 mg/kg per day vitamin E was used for 30 days, the authors found an improvement in the antitumour activity of doxorubicin, as indicated by an increase in the average survival of the animals, an increase in the number of animals that survived long term and a decrease in bodyweight wasting induced by doxorubicin treatment. Similarly, Shimpo *et al.*[6] reported that ascorbic acid had no effect on the antitumour activity of doxorubicin in mice inoculated with leukaemia Ll 210 (doxorubicin was administered as a single dose of 5 mg/kg) or Erlich ascites carcinoma (three i.p. administrations of 0.5 mg/kg), but that it significantly increased the survival of animals treated with doxorubicin. In these experiments, ascorbic acid (2 g/kg) was given daily. In the same series of studies, the authors provided evidence that ascorbic acid and some derivatives, such as ascorbyl palmitate or 2-*o*-octadecylascorbic acid, could reduce lipid peroxidation and other markers of doxorubicin toxicity, which was administered at several doses, in the heart of guinea-pigs and mice. These results prompted the authors to suggest that combined treatment with doxorubicin and ascorbate or its derivatives could be clinically effective in cancer patients. However, the present study is the first to evaluate the possible effect of concomitant supplementation of vitamins C and E, once it was well established that the beneficial effect of each vitamin alone in reducing the toxic effects of doxorubicin treatment did not change its antitumour properties.

Previous studies have shown a significant increase in lipid peroxidation products in the brain, liver, lung and kidney tissues after a single dose of doxorubicin,[10–14,32,35] suggesting nephrotoxicity, so we assayed biochemical parameters associated with renal function. Urea and creatinine concentrations were increased in the plasma of animals in the doxorubicin-treated group. This confirms other studies[34,35] and reinforces the hypothesis of the compromise of kidney function promoted by nephrotoxicity. Conversely, supplementation with creatine and vitamins C and E was efficient in reverting the increase of these metabolites caused by doxorubicin. The creatine-supplemented group showed a decrease in urea concentration in relation to the doxorubicin-treated group, whereas supplementation with the vitamin mix was more efficient in decreasing both urea and creatinine concentrations, suggesting a greater nephroprotector effect of this solution compared with creatine.

We conclude that the two supplementation protocols evaluated in the present study are efficient in decreasing the cytotoxic effects of doxorubicin and increase survival after doxorubicin treatment. In addition, these protective effects are more noticeable in animals supplemented with the mixture of vitamins C and E.

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Reevaluation of Nitrate and Nitrite Levels in the Human Intestine[1]

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ABSTRACT

Analyses of human fecal and ileostomy samples by a method that is sensitive and free from interferences indicate that nitrate and nitrite levels in the intestine are lower than reported previously. Fecal nitrate and nitrite concentrations ranged from 0 to 14 μmol/kg (0 to 0.9 ppm) and 5 to 19 μmol/kg (0.3 to 0.9 ppm), respectively. Ileostomy samples contained from 0 to 7 μmol/kg (0 to 0.4 ppm) and 0 to 15 μmol/kg (0 to 0.7 ppm) for nitrate and nitrite, respectively. We also showed that, when deliberately added to feces samples, nitrate and nitrite were destroyed during a two-hr incubation period in a reaction that depended on the presence of microorganisms. The results suggest that conditions in the lower gastrointestinal tract favor denitrification, not nitrification as had been proposed previously.

INTRODUCTION

Endogenous synthesis of nitrate and nitrite from reduced forms of nitrogen in the human intestine has recently been proposed by Tannenbaum *et al.* (15). This proposal has important implications for cancer etiology, since nitrite is a precursor of carcinogenic *N*-nitroso compounds that may be formed *in vivo*. The direct evidence cited by Tannenbaum *et al.* (15) for nitrification by intestinal microorganisms derives from their findings of relatively high levels of nitrate and nitrite in feces and of nitrite in ileostomy fluid. In support of these results, Gomez *et al.* (4) reported the isolation of several organisms from the human intestine that are able to oxidize nitrogenous compounds to nitrite *in vitro*. Several other workers, however, have failed to detect nitrate or nitrite in human ileostomy fluid or feces samples (5, 6, 8). Witter *et al.* (22) have pointed out several reasons why heterotrophic nitrification is unlikely to occur in the gastrointestinal tract. In particular, they cite the lack of oxygen in the distal ileum and colon and the sparseness of nitrifying bacteria in the upper intestinal tract.

Indirect evidence for endogenous nitrate and nitrite synthesis in the intestine derives from nitrate balance studies in humans in which nitrate excretion in urine has been reported to exceed dietary intake levels by factors of 2 to 60 (9, 10, 14). Others, however, have reported that nitrate in urine is not in excess of intake levels (1, 6, 11). From measurements of the body distribution of nitrate labeled with ^{13}N, Witter *et al.* (21) have concluded that humans have the capacity to store nitrate in their bodies; these workers suggest that urinary, ileal, and fecal nitrate values might be explained by depletion of body stores, passage of nitrate down the intestine, or secretion of nitrate into the intestinal lumen. Some of these conclusions may be erroneous, however, for reasons related to their experimental design, particularly the short half-life of ^{13}N compared to the much longer time required for distribution and clearance of administered nitrate and the inability to distinguish between ^{13}N-labeled nitrate and its reaction products (14).

In order to resolve some of these disparities in the literature concerning the biodynamics of nitrate and nitrite, we have developed an analytical procedure for measuring nitrate and nitrite concentrations in feces that is sensitive and free from the interferences that are inherent in methods that do not include extensive cleanup of the sample. We have used the method to measure nitrate and nitrite levels in fresh human feces and ileostomy fluid and to determine the stability of the ions during incubation with fecal organisms.

MATERIALS AND METHODS

Feces samples were donated by healthy males, 18 to 37 years old, who were eating a free-choice, Western-style diet. Ileostomy fluid samples were donated by males and females, 20 to 55 years old, who had either a conventional or Kock ileostomy following colectomy for ulcerative colitis (3) but who were otherwise in good health. Samples were collected at various times throughout the day. Three of the donors with conventional ileostomies were taking medication (Naprosyn; Lomotil and cimetidine; and prednisone and acetaminophen, respectively). All others were free of medication.

Our analytical procedures for nitrate and nitrite were based on the methods of Sen and Donaldson (12) and Sen and Lee (13) for determination of nitrate and nitrite in foods. During the course of this work, we found that both nitrate and nitrite are rapidly destroyed by fecal microorganisms. In order to minimize this destruction, analysis was begun on both fecal and ileostomy samples within 2 min of excretion. Thirty to 40 g of sample were diluted 6-fold with 0.4 M NaOH to stop loss of nitrate and nitrite. The slurry was blended for 5 min in a VirTis homogenizer. Sixty ml of the slurry were transferred to a flask and heated for 10 min at 60°. Ten ml of 1.4 M zinc sulfate were added to precipitate protein, and the sample was heated at 60° for a further 10 min. After cooling to room temperature, the sample was filtered using a glass microfiber filter (GF/A; Whatman, Inc., Clifton, N.J.). The residue was washed with 20 ml water, and the filtrates containing the extracted nitrate and nitrite were combined.

A 6- x 1-cm anion-exchange column was prepared with AG 1-X8 resin, 100 to 200 mesh, chloride form (Bio-Rad Laboratories, Richmond, Calif.). The column was washed first with 50 ml 4 M NaCl to remove any contaminating nitrate and nitrite

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ions and then with 50 ml water. After the entire filtrate was added to the column, it was washed with 25 ml water, and the nitrate and nitrite were eluted with 20 ml 4 M NaCl. After pretreatment of a C_{18} Sep-Pak cartridge (Waters Associates, Milford, Mass.) with 5 ml methanol and 10 ml water, the entire 20-ml sample was passed through the cartridge and collected in a calibrated vessel. The cartridge was then washed with 4 ml water, and the combined eluate was adjusted to 25 ml with water. Ten ml of this purified solution, representing 4 g of the original sample, were analyzed for nitrite by means of the Griess reaction, as described by Sen and Donaldson (12). Total nitrate and nitrite were determined after reduction of another 10-ml aliquot of the purified sample on a spongy cadmium column (12). The nitrate concentration was calculated as the difference between the total nitrate plus nitrite value and the nitrite value. Following the Griess reaction, absorbances at 550 nm were measured against reagent blanks that had been taken through the same procedures as the samples. A standard curve was constructed by adding nitrite solutions of known concentrations to other reagent blanks that had also been taken through the entire procedure. All the results are expressed as μmol of nitrate or nitrite per kg, wet weight, of sample.

To determine percentage recoveries, samples were homogenized with a small volume of water immediately after collection and were divided into 2 or more aliquots, each containing 10 g of the original sample. Standard nitrate and/or nitrite solutions were added to some aliquots (spiked samples), while water was added to one or more of the aliquots which served as controls. Sodium hydroxide solution was then quickly added, and the samples were analyzed as described above.

In order to measure the stability of nitrate and nitrite in feces, fresh feces were mixed with an equal weight of deaerated 0.01 M phosphate buffer, pH 7.4, containing 0.15 M sodium chloride and were homogenized briefly in a VirTis homogenizer. Ten-g samples of the slurry, representing 5 g of feces, were weighed into Erlenmeyer flasks to which were added 2.5 μmol of either sodium nitrate or sodium nitrite (yielding final concentrations of 500 μmol/kg based on the original feces). The flasks were sealed with rubber septa, and humidified, filtered nitrogen or air was flushed continuously into the headspace of each flask via a hypodermic needle, with another needle acting as an outlet for the gas. The flasks were incubated in a metabolic shaker at 37°. At appropriate times up to 2 hr, flasks were removed and analyzed for nitrate and nitrite as described above. Feces samples that had been autoclaved at 120° under pressure for 15 min served as controls.

RESULTS AND DISCUSSION

Numerous methods have been described for the analysis of nitrate and nitrite in environmental and biological samples (16). Methods for nitrate analysis are particularly susceptible to interferences and are generally unreliable (16). Difficulties in the measurement of nitrate and nitrite in the gastrointestinal contents of rodents using the colorimetric Griess reaction have been noted by Witter and Balish (19). Sen and Lee (13) have recently overcome problems associated with analysis of nitrate and nitrite in complex foodstuffs by utilizing an extensive cleanup of samples to remove interferences before using the Griess reaction. In view of their success, we chose to follow a similar analytical scheme for feces and ileostomy fluid. After deproteinization, anion-exchange chromatography, and treatment with reverse-phase adsorbent, our samples were free of both colored materials (sample absorbance at 550 nm was less than 0.002 absorbance unit) and chemicals which might interfere with the Griess reaction itself (as judged by the complete recovery of azo dye if nitrate or nitrite were added after the cleanup steps). In contrast, we found that an aqueous feces

Table 1

Recoveries of nitrate and nitrite added to feces and ileostomy fluid

	Spiking level (μmol/kg)		Measured level (μmol/kg)		Recovery %[a]	
Sample	Nitrate	Nitrite	Nitrate	Nitrite	Nitrate	Nitrite
Feces						
1	0	0	4	12		
1	0	0	4	11		
1	0	20	2	28		82
1	0	20	1	29		87
1	20	0	21	12	85	
1	20	0	20	10	80	
1	20	0	17	12	65	
2	0	0	1	8		
2	20	20	19	21	90	65
3	0	0	1	7		
3	50	50	32	43	62	72
4	0	0	7	10		
4	50	50	44	45	74	70
5	0	0	11	7		
5	25	0	36	6	100	
5	50	0	46	4	70	
Av. recoveries					78.2 ± 4.6%[a]	75.2 ± 4.0%
Ileostomy						
K[b]	0	0	5	2		
K	50	50	44	42	78	80
C	0	0	2	6		
C	50	100	39	90	74	84
Av. recoveries					76.0 ± 2.0%	82.0 ± 2.0%

[a] Mean ± S.E.
[b] Kock ileostomy fluid; C, conventional ileostomy fluid.

extract that had had no cleanup gave virtually complete inhibition of azo dye formation when spiked at the level of 100 μmol/kg (the azo dye in this case was measured by high-performance liquid chromatography using a C_{18}-μBondapak column, eluted with 35% acetic acid, pH 2.5).

Percentages recoveries of nitrate and nitrite added to feces and ileostomy fluid are shown in Table 1. Low spiking levels of nitrate and nitrite were used (20, 25, and 50 μmol/kg or 0.9 to 3.1 ppm), since the levels found in feces and ileostomy fluid were also low. The somewhat variable recovery values result from the use of these low spiking levels.

Table 2 illustrates the levels of nitrate and nitrite that we analyzed in 9 feces and 8 ileostomy samples. These values are corrected for percentage recovery, using the average recoveries for each ion and sample type (shown in Table 1) as correction factors. Based on the variation among replicate samples found in Table 1 and on the SE in the correction factors, the error in both the nitrate and nitrite values of Table 2 is estimated to be ± 5 μmol/kg. Values of less than 5 μmol/kg were not considered to be significantly different from 0 and have been reported as not detected. Small but detectable concentrations of nitrate were found in 6 of 9 feces samples. All of the feces samples contained small concentrations of nitrite. Our results, which indicate maximum concentrations of 14 μmol/kg for nitrate and 19 μmol/kg for nitrite, are to be compared with the values of Tannenbaum *et al.* (15) for similar samples that range from less than 0.5 to 128 μmol/kg for nitrate and 13 to 112 μmol/kg for nitrite [these values have been corrected from a dry to a wet weight basis for comparison with our results by applying a factor of 3.8 that we have determined is the median value of the wet/freeze-dried weight ratio (range, 2.6 to 6.4)[3]]. Our maximum values for nitrate and nitrite in feces would correspond to fecal excretion rates of 0.09 mg nitrate per day and 0.09 mg nitrite per day, assuming a feces output rate of 105 g/day (2). White (17, 18) has estimated that the average North American ingestion rates of dietary nitrate and nitrite are 99.8 and 2.6 mg/day, respectively (more recent data suggest that this nitrite value may be somewhat overestimated[4]). Therefore, the maximum estimated fecal excretion rates for nitrate and nitrite are far lower than the normal dietary intake rates.

Our results for analysis of ileostomy samples also differ from those of Tannenbaum *et al.* (15), particularly with regard to nitrite levels. The maximum nitrite value in our experiments was 15 μmol/kg wet weight (Table 2). In contrast, the nitrite values previously reported for similar samples range from 37 to 162 μmol/kg, wet weight [using a wet/freeze-dried weight ratio of 9.5 as a correction factor for these samples (7)]. While Tannenbaum *et al.* (15) did not detect any nitrate in ileostomy fluid samples, we detected nitrate in 3 of 8 samples. The reasons for the discrepancy between our results and those of Tannenbaum *et al.* (15) are not clear at this time, but we believe that their analytical method may not have adequately removed interferences. Our data also suggest that there are no differences with respect to nitrate and nitrite values between conventional or Kock ileostomy fluid or between patients receiving or not receiving medication. It should be noted that ileostomy fluid is ileal fluid that has been retained in a sack for varying periods of time and is obtained from persons whose health status is not normal. Nitrate and nitrite levels found in ileostomy fluid, therefore, may not represent the levels in the normal ileum.

Nitrate and nitrite in the intestine are undoubtedly undergoing a continuous succession of changes, both ions being either generated by nitrification, destroyed by reductive metabolism or chemical reaction (in the case of nitrite), or transferred across the intestinal wall either to or from the bloodstream. In order to better understand the fate of nitrate and nitrite in the intestine, we have examined both the anaerobic and aerobic incubation of feces samples to which nitrate or nitrite have been added. Chart 1 illustrates that, at 37° under anaerobic conditions, nitrate and nitrite are rapidly lost during incubation with feces. The nitrate and nitrite disappearances were observed to obey approximately first-order kinetics, with half-lives of about 20 and 5 min, respectively. There were only slight losses of nitrate and nitrite during anaerobic incubation with samples that had been autoclaved. Incubation of samples into which air was passed instead of nitrogen gave almost identical results to the anaerobic incubations (although in the aerated

Table 2

Levels of nitrate and nitrite in feces and ileostomy fluid

Values corrected for percentage recovery; estimated error is ±5 μmol/kg.

Sample	Nitrate (μmol/kg)	Nitrite (μmol/kg)
Feces		
1	6	16
1	5	14
2	ND[a]	11
3	ND	9
4	8	13
5	14	9
6	5	19
7	5	13
8	ND	9
9	9	5
Ileostomy		
K1	6	ND
K2	5	9
K3	ND	9
K4	ND	10
C1	ND	7
C2	ND	5
C3	ND	15
C4	7	15

[a] ND, not detected (below the detection limit of 5 μmol/kg); K, Kock ileostomy fluid; C, conventional ileostomy fluid.

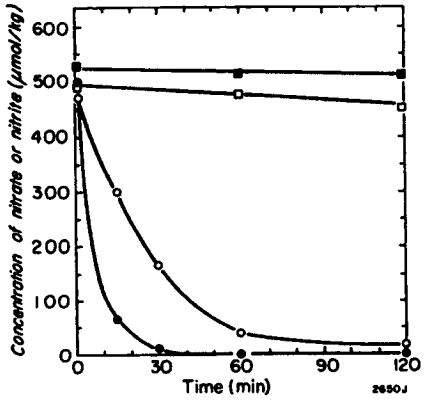

Chart 1. Concentrations of nitrate and nitrite following incubation with human feces under anaerobic conditions. ○, fresh feces plus nitrate, 500 μmol/kg; □, autoclaved feces plus nitrate, 500 μmol/kg; ●, fresh feces plus nitrite, 500 μmol/kg; ■, autoclaved feces plus nitrite, 500 μmol/kg.

[3] P. W. Dion, Ontario Cancer Institute, unpublished data.
[4] J. Birdsall, American Meat Institute, unpublished data.

samples, the majority of fecal organisms, other than those at the surface, was probably still in a predominantly anaerobic environment). In 2 other experiments, feces samples to which no nitrate or nitrite had been added were incubated. In these cases, the endogenous nitrate and nitrite levels decreased with time at what appeared to be a slower rate than was observed for added nitrate and nitrite, although an accurate kinetic analysis was not possible, since all measurements were made near the detection limit of the analytical method.

The rapid loss of added nitrate during its incubation with feces is probably due to the presence of facultative and anaerobic microorganisms which possess nitrate reductase activity. Nitrite, which is the expected first product of nitrate reduction, was not detected, however, in samples incubated with nitrate. Presumably, nitrite was formed in these samples but was not detected because nitrite itself is very rapidly lost, as shown in Chart 1. In feces samples incubated with added nitrite, no nitrate was detected. These results suggest that, under the conditions of our experiment, nitrite is not oxidized to nitrate as proposed by Tannenbaum *et al.* (15) but that both nitrate and nitrite are reduced to lower oxidation states of nitrogen by fecal microorganisms. Our results for human feces are consistent with animal data obtained by Witter and Balish (19) who observed that both nitrate and nitrite are rapidly destroyed when incubated with the intestinal contents of conventional-flora Sprague-Dawley rats. In their study, nitrate and nitrite were also incubated with the intestinal contents of germ-free rats, and no destruction of the ions was observed, further indicating the requirement for microorganisms in this process.

With such a rapid rate of loss of added nitrate and nitrite, we were surprised that we were able to detect even low levels of the ions in feces to which no nitrate or nitrite had been added. One explanation for these low levels is that some dietary nitrate and nitrite pass directly through the gastrointestinal tract but are not available to the intestinal microorganisms for enzymatic reduction. The unavailability of these ions might be caused by their containment in undigested food particles, ionic binding to macromolecules, or covalent binding in compounds such as nitrate and nitrite esters. In these ways, the ions may not be destroyed during passage through the intestine but are nevertheless released and detected during the analytical procedure. Alternatively, nitrate and nitrite that have been absorbed from the stomach or upper intestine may reenter the lower gastrointestinal tract from the bloodstream (20).

In summary, our results, obtained using a sensitive analytical procedure with high recovery values, indicate that the nitrate and nitrite levels in human feces samples are generally less than 20 μmol/kg, wet weight (1 ppm). The levels of nitrate and nitrite in ileostomy fluid are also very small. Despite the presence in human feces of organisms capable of heterotrophic nitrification *in vitro* (4), the overall conditions in the lower gastrointestinal tract appear to strongly favor nitrate and nitrite reduction.

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EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Health Opinions
Unit C3 - Management of scientific committees II; scientific co-operation and networks

Scientific Committee on Food

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Opinion of the Scientific Committee on Food on safety aspects of creatine supplementation

(Adopted by the SCF on 7 September 2000)

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Opinion of the Scientific Committee on Food on safety aspects of creatine supplementation

(Adopted by the SCF on 7 September 2000)

Introduction

A report on "the composition and specification of food intended to meet the expenditure of intense muscular effort, specially for sportsmen" was adopted by the SCF in June 2000. This report addressed efficacy but not safety issues. One aspect of the report concerned creatine and it was concluded that creatine supplementation can lead to improved exercise performance in events requiring explosive, high energy activity, especially of a repeated nature. The Committee has been asked to comment on the safety of recommended creatine supplementation regimes, since some of these involve consumption of relatively large doses. The Committee wishes to emphasise that this separate opinion on the safety of creatine should not be taken to imply that safety aspects of other components of sports foods may not also need to be addressed.

It is understood that the use of oral creatine supplements is common among professional and amateur sportsmen and women. Its use has spread to college athletes, recreational athletes and even children. Because creatine is considered a nutritional supplement, it is currently freely available from pharmacies, health food stores and supermarkets. In this context, a question was raised on the safety of the recommended ingestion protocols (see below). A Medline search (1998-March 2000 period) has been made, using the key words "creatine" and "safety", to review the current scientific information on creatine supplementation.

Creatine (N-(aminoiminomethyl)-N-methyl glycine) occurs naturally in foods such as meat, fish and other animal products. A typical diet includes 1-2 grams of creatine daily, but it may also be formed endogenously by liver, kidney and pancreas from the amino acids Gly, Arg and Met at the rate of 1-2 g/day (2, 10).

Muscle stores of creatine can be maximised by a regimen that initially loads the muscle and then maintains a maximal increased state when lower doses are ingested (8, 3). The dosing regimens suggested by the manufacturers are 20 g/day for 3 to 7 days and then 2-5 g/day as a maintenance dose. Loading doses in the range of 10-50 g/day for 5-7 days are currently used although it is accepted that the same effect can be achieved by a 3 g/day dose over a more prolonged period (28 days) without a loading phase (10, 11, 13).

It has been shown that oral creatine supplementation (at the doses described above) produces a significant increase (about 10-20 %) in skeletal muscle creatine, which is predominantly free creatine, although 20-40 % of the increase can be in the form of phosphocreatine (3). Simultaneous consumption of carbohydrate with creatine further increases the creatine and phosphocreatine levels in muscle and also can facilitate muscle glycogen storage.

In a healthy subject of 70 kg with a total creatine pool of 120 g (95% in skeletal muscle), the daily turnover is about 2 g. Taking into account the gastrointestinal absorption, this amount can be obtained by an intake of about 2-3 g/day (1, 13). Creatine is transported to muscle and nerve and crosses the cell membrane via a specific transporter system against a 200:1 gradient (8). Creatine is eliminated by its irreversible conversion to creatinine at a rate of about 1-2 g/day (1, 9). This is the amount of creatine that needs to be replaced each day, either by endogenous synthesis or from dietary sources. Thus, the loading dose of oral creatine taken by many sportspeople for short periods of time represents about 10 to 20 times the daily turnover.

Safety considerations

Creatine appears to be well tolerated in short term human trials. Dozens of clinical trials have been conducted (see references in 2, 10, 13), mostly in highly trained athletes, and no adverse effects have been noted, although many trials reported increased body mass. However, the primary objective of these studies was to assess the effects on exercise performance and not adverse effects. The assumption that short term use (fewer than 28 days) at recommended doses has not been shown to cause significant adverse effects is based on these studies, but they have involved small numbers of subjects and there are no sample size calculations to indicate the limitations on the power of the studies. Other reports have linked creatine supplementation to weight gain, cramping, dehydration, torn muscles, gastrointestinal distress and dizziness (2, 8, 12, 13). (See also annex I).

Because of the high nitrogen content of creatine, the potential for renal dysfunction in athletes treated with creatine has been raised. Two cases of decreased renal function have been described so far (5, 7). However one study (4) found no detrimental effects on the kidney in eight young men and one woman after short-term, medium-term, or long-term oral creatine supplementation (10 months to 5 years). In addition, although creatine is normally found not only in skeletal muscle but also in cardiac muscle, brain and testes, these three areas remain essentially unstudied (3, 12).

Another question that may be raised is whether inhibition of endogenous creatine synthesis, which is produced by creatine administration, is reversed when creatine supplementation is terminated. There is also some concern about taking creatine supplements if sportspeople become dehydrated or in conjunction with other supplements, since possible interactions are unknown (8). In addition, because marketed creatine products do not meet the same quality control standards of pharmaceuticals, there is a potential concern about impurities of unknown toxicity, particularly dicyandiamide and dihydrotriazines. The possibility has also been raised that doses higher than those recommended on the labelling may be consumed (2). These authors also pointed out that potential interactions of most supplements, including creatine, with drugs have not been studied (2).

Conclusions

It can be concluded that although many efficacy trials have studied the effects of creatine, large-scale, well-controlled studies are lacking. Available results observed in highly trained athletes cannot necessarily be extrapolated to the general public. Little information exists on the short-term or long-term safety of creatine and evidence of adequate quality control of the commercially marketed creatine is lacking and adequate specifications for food grade materials should be developed.

Although no important adverse effects have been reported in the efficacy trials, such evidence is insufficient to provide reassurance about the safety of creatine supplementation involving high loading doses: there are doubts about safety in relation to kidney function; studies on tissues in which creatine is known to concentrate are lacking; effects on endogenous creatine synthesis upon cessation of supplementation are also not well studied. For these reasons the Committee considers that high loading doses should be avoided. Consumption of lower doses of up to 3 g/day are similar to the daily turnover rate of about 2 g/day and are unlikely to pose any risk.

Future studies should evaluate short- and long-term effects of oral creatine on renal and hepatic systems as well as those organs where creatine plays a metabolic role. Such studies should include people who are not highly trained.

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ANNEX 1

A summary of creatine metabolism in various organ systems and concerns regarding the effects of oral creatine supplementation was reported by Juhn and Tarnopolsky (12) as follows:

Organ system/Effect	Comments
Cardiovascular	Effect of long term oral creatine on cardiac muscle creatine concentration and cardiac function is unknown. No effects seen in short term use (10 days).
Gastrointestinal	Diarrhoea and gastrointestinal pain anecdotally reported, but no direct relationship established.
Liver	Studies up to 8 weeks show minimal or no liver enzyme elevation. Concern exists regarding the reversibility of the suppression of endogenous creatine synthesis after long-term use.
Musculoskeletal	Because of water retention in the muscle cell, there is theoretical concern about muscle cramps and tears, but causal relation not established.
Neurologic	Creatine is naturally found in brain tissue. The effects of oral creatine on brain concentrations is unknown.
Oncologic	Creatine and phosphocreatine/creatine kinase system may influence cellular oncogenesis. Long-term studies would help determine if oral creatine supplementation is beneficial, detrimental, or has no effect on healthy subjects.
Paediatric/adolescent	Theoretical concerns exist regarding extra load placed on developing kidney/other organs and the effects of creatine on muscle/bone junctions in the skeletally immature.
Renal	Urinary excretion of creatine increases up to 90-fold, though glomerular filtration rate is unchanged, at least during the 5-day loading phase. Elevation of serum and urinary creatinine also occurs, but generally small in studies of less than 28 days. Concern lies with unknown effects of longer-term supplementation (*).
Reproductive organs	Creatine is normally synthesised in the testes by the Sertolli cells. Creatine and Phosphocreatine are involved in sperm metabolism, but no studies exist on the effects of oral creatine supplementation. As with liver, concern regarding reversibility of the suppression on endogenous creatine synthesis.
Weight gain	Proven to occurs in many studies. Initially caused by water retention. With prolonged use, increased muscle synthesis may also occur; this is being investigated.
Dehydration	Intracellular fluid retention in the muscle cell may predispose to dehydration, but studies are lacking. Proper hydration during supplementation is encouraged.
Long term effects	Unknown in any organ. Studies involving 12 months or more are needed, preferably with larger sample sizes than previous studies.

(*): One limited long term study (4) showed no adverse effects

EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

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SCIENTIFIC COMMITTEE ON FOOD

SCF/CS/NUT/SPORT/5 Final (corrected)
28 February 2001

Report of the Scientific Committee on Food on composition and specification of food intended to meet the expenditure of intense muscular effort, especially for sportsmen

(Adopted by the SCF on 22/6/2000,
corrected by the SCF on 28/2/2001)

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i Index
ii Definitions and abbreviation

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Athletes	Generic term used for sportsmen and sportswomen
CES	Carbohydrate-electrolyte solution
CHO	Carbohydrate
Ergogenic	Tendency to increase physical work or mental work performance.
FIFA	Fédération Internationale de Football Association
Glycemic Index	Blood glucose response to a 50 g portion of the food as area under the curve over 3 hours, expressed as percentage of the response to the same amount of ingested glucose over the same period of time
IAAF	International Federation of Athletic Associations
Isotonic	Refers to osmolality of body fluids (297 mOsm/kg water)
NPU	Net Protein Utilisation
Osmolality	The number of particles (molecules or ions) per unit of molecular weight of undissociated solute. For example: 180 g of glucose in 1-kg water is 1 osmol with an osmolality of 1 Osm/kg.
PRI	Population Reference Intake

Report of the Scientific Committee on Food on composition and specification of food intended to meet the expenditure of intense muscular effort, especially for sportsmen

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1. Executive summary

Council Directive 89/398/EEC on foodstuffs intended for particular nutritional uses, as amended by Council Directive 1999/41/EC, foresees the adoption, by the Commission, of a specific directive on foodstuffs for particular nutritional uses intended to meet the expenditure of intense muscular effort and especially for sportsmen. In order to prepare this specific directive the Commission asked the Scientific Committee for Food (SCF) for advice on the nature, the essential composition where necessary, and any other specific requirements concerning the labelling and the appropriate use of such foodstuffs.

The Committee reviewed the scientific literature in the area of sport nutrition as well as a number of consensus reports that were prepared by various sport organisations and came to the conclusion that the concept of a well-balanced diet is the basic nutritional requirement for athletes. Nevertheless, taking the aspects of intense muscular exercise in consideration such as intensity, duration and frequency as well as specific constraints like time and convenience, individuals can benefit from particular foods or food ingredients beyond the recommended dietary guidelines for the general population.
As the increased energy need of these individuals is the most apparent difference, the food intake is higher. This can lead to differences in food choice and eating pattern as well as gastro-intestinal distress. Specially adapted nutritious foods or fluids may help to solve specific problems so that an optimal nutritional balance can be reached. These beneficial effects are not only limited to athletes who are taking regular intense prolonged muscular exercise, but are also intended for other target groups, for example for occupational jobs with hard physical work or with extreme environmental conditions, as well as for individuals with irregular physical high intensity or fatiguing leisure time activities.

In relation to these general considerations, four food categories have been identified, reviewed and where applicable, essential requirements were formulated.

- Carbohydrate-rich energy food products

Consensus has been reached about the essential role of carbohydrate intake in relation to physical performance during all types of exercise, generally lasting longer than one hour. This knowledge is based on the importance of increased body glycogen stores in liver and muscle for sustaining prolonged heavy exercise, as well as the direct relationship between the level of carbohydrate intake and the resynthesis of muscle glycogen after exhausting exercise.
Ad libitum eating during 24 h after prolonged heavy exercise may lead to an inadequate intake of energy, especially carbohydrates and consequently, a sub-optimum recovery. Therefore when athletes have only 24 h to recover from prolonged heavy exercise, optimal carbohydrate intake should be guaranteed by specific instructions regarding the timing and choice of carbohydrate intake by food and/or carbohydrate rich food products. High glycaemic index carbohydrate foods are recommended and they should provide 10g per kgbw during the 24-h recovery. The refuelling should begin immediately after the exercise bout when athletes should consume up to 1 g/kgbw of carbohydrate and then about 0,5 g/kgbw at hourly intervals until the next meal, which should be made up of high glycaemic index carbohydrate foods.
In this respect all types of bio-available carbohydrates that increase blood glucose concentration effectively are suitable. Besides the high carbohydrate/low fat dietary guidelines, especially developed carbohydrate-rich energy food products can be of benefit in reaching an adequate carbohydrate intake

- Carbohydrate-electrolyte solutions (C.E.S.)

The two factors that have been considered to contribute most to the onset of fatigue in exercise are the depletion of the body's carbohydrate reserve and the onset of dehydration, as a consequence of the loss of water and electrolytes in sweat.
Compared to water as a control drink, a substantial body of scientific evidence supports the suggestion that during prolonged exercise drinks containing carbohydrates and electrolytes, in particular sodium, improve the performance.
The optimum carbohydrate concentration in the drink depends on a number of factors, among others the need for water (hot/cold conditions) and the intensity and type of exercise (gastro-intestinal absorptive capacity, osmolality (rate of gastric emptying as well as water absorption in the small intestine), type of carbohydrate simple vs. polymers). Therefore a range from 80-350 kcal (335 - 1470 kJ) CHO/1000 ml CES drink is advised. The only electrolyte added to drinks consumed during exercise that is known to confer physiological benefit is sodium. A sodium concentration of 20-50 mmol/l (460 - 1150 mg/l) will stimulate carbohydrate and water uptake maximally in the small intestine and will help to maintain extracellular fluid volume. The

evidence to support the inclusion of other components as essential ingredients, is not at present convincing.

- Protein and protein component

Athletes continue to believe, as did the Olympians of antiquity, that extra protein intake is essential for maximal performance. There is not much scientific evidence available to support this.

Endurance athletes have a modest increase in protein requirements and, therefore, the recommended daily intake is increased to 1.2 - 1.4 g per kgbw per day. A diet containing 10-11 % En protein meets this modest increase, as the daily energy needs may be two to three-fold higher than those of non-athletic subjects. The use of protein-carbohydrate solutions or protein-carbohydrate rich solid food products in the post-exercise period may help to rapidly re-synthesis glycogen stores that were lost during the exercise.

The protein requirement for strength athletes, who have trained for years, is not higher than 1.0 to 1.2 g per kgbw per day. Novice athletes involved in strength training programs have a marginally higher protein requirement and their recommended intake is therefore increased to 1.3 to 1.5 g per kgbw per day. A diet containing 10-12 % En protein of mixed quality may not contain enough protein to meet this temporary need if the total energy intake is relatively low. In addition there is no scientific evidence at all for further increases in protein intake with protein supplements to levels of 3-6 g per kg bw per day, as frequently occurs in practice. Also the use of supplements of free amino acids has not beneficial effects on the whole body and protein synthesis when compared to the use of a balanced protein in a mixed meal.

- Supplements

For micronutrients there is a scientific consensus that with an adequate dietary intake, there is no further need for additional supplementation for essential micronutrients such as minerals, trace elements and vitamins. In the case of restricted food intake, as is frequently observed in weight related sports, micronutrient intake could become marginal or deficient, which would justify supplementation. Intake of a number of minerals and vitamins such as magnesium, calcium, zinc and the anti-oxidants vitamins C, E as well as carotenoids, have been suggested to be critical in relation to physical performance. So far, scientific evidence is lacking or inconsistent in supporting recommendations for nutritional intakes beyond the accepted dietary guidelines. The upper safe levels of vitamin- and mineral intake are at present under consideration of the SCF and have not been reviewed in this report.

Finally, a number of food components have been reviewed since they are often related to physical performance. So far, only for caffeine and creatine is there scientific data to show that

they have an ergogenic effect. For caffeine levels of 3 to 8 mg/kgbw improve short-term high intensity exercise as well as endurance performance. Creatine intake levels of 2 – 3 g per day have been shown to be effective in increasing total muscle creatine and in improving performance of a short term high intensity exercise.

The Committee wishes to stress that this report is dealing with the physiological needs and appropriate uses of food and food ingredients to meet the expenditure of intense muscular effort. The safety aspects of high level of intake of certain compounds such as free aminoacids are not taken into consideration. Upper safe levels of vitamin and nutrient intakes are at present under consideration of the SCF. The Committee has adopted opinions on the safety of caffeine in the past (Opinion on caffeine, taurine and D-glucurono- gamma -lactone as constituents of so-called "energy" drinks, expressed on 21 January 19991). The Committee considers the safety aspects of creatine supplementation in a separate report.

[1] available on the internet at the SCF pages

2. Terms of reference

To advise on the nature, the essential composition, and, where necessary, any specific requirements concerning the labelling and appropriate uses of foods intended to meet the expenditures of intense muscular effort, especially for sportsmen.
The Commission intends to use this advice to prepare a specific directive on these foods as foreseen in the framework Council Directive 89/398/EEC of 3 May 1989 as amended by Council Directive 1999/41/EC on the approximation of the laws of Member States relating to particular nutritional uses.

3. Consulted experts and documents

In drafting the opinion, the Working Group on Nutrition of the SCF was enlarged with four experts:

- Dr. A. Berg, Dept. of Sport Medicine, University Freiburg, Freiburg, Germany
- Prof. Dr. R.J.M. Maughan, Environmental and OccupationalMedicine University of Aberdeen, Aberdeen, United Kingdom
- Dr. A.J.M. Wagenmakers, Nutrition and Toxicology Research Institute NUTRIM, University of Maastricht, Maastricht, The Netherlands
- Prof. Dr. C. Williams, Dept. of Physical Education and Sports Sciences, Loughborough University, Loughborough, United Kingdom.

The Committee examined many published papers in the area of sports nutrition, including the proceedings of an IOC (International Olympic Committee) International Scientific Consensus "Food Nutrition and Sport Performance" in 1991 [49] the FIFA Consensus "Food, Nutrition and Soccer Performance" in 1994 [50] and the IAAF Consensus "Current Issues in Nutrition and Athletes" in 1995 [104] and had a meeting with some experts from the IDACE.
The Committee also took notice of the report "Sports food" from the Association of Dietetic Food Industry of the EC [73].

4. General considerations

Nutrition significantly influences physical performance. This relationship is even more clearly demonstrated during intense muscular exercise. As a consequence in competitive sports disciplines, nutrition has become important for performance, now those athletes have reached the limits of training volume and training intensity. Among athletes and exercise physiologists this has led to a

renewed interest in the role of nutrition and the influence of gastrointestinal problems on physical performance and well being.

A realistic conclusion in the literature, is the concept that a well-balanced diet is the basic nutritional requirement for athletes. In addition to this, specific nutritional requirement may arise depending on particular physiological conditions, which are the consequence of the athletic training and performance. The question remains to what extent these specific nutritional needs differ from the dietary guidelines for the general population.

This question should not be limited to athletes taking regularly intense prolonged muscular exercise, but also to other target groups involved in regular or irregular intense muscular exercise in a number of occupational jobs, such as rescue services, military services and industrial sites with extreme environmental conditions (high/low temperature). There are also large numbers of individuals who are physically active on a recreational basis, sometimes in irregular high intensity or fatiguing exercise. In a recent pan – EU survey [52] 30 to 40 % of the adult EU population spent more than 8 h per week being physically active. A proportion of this group, including those involved in sport should be regarded as a potential target group that can benefit from specific sports food to support health and performance and to minimise the risk of unwanted outcomes such as injury.

In the past three decades the question about extra nutritional needs beyond a well-balanced diet has been extensively addressed within the nutritional and exercise sciences. This research was also stimulated by the rapidly increasing market for sports foods, in particular the carbohydrate-electrolyte solutions (CES). At the same time, an increasing interest in functional food ingredients, which may affect body functions in a positive way, has arisen. In this respect sport nutrition is the good example of scientifically developed concepts in the area of foods or food ingredients intended to influence particular physiological functions.

For the evaluation of the scientific background to the use of particular foods for intense muscular exercise, one should address in the first place the issue of beneficial effects of food or food ingredients beyond the normal recommended nutritional intake on physical performance, taking specific constraints like time and convenience into consideration.

Several aspects of intense muscular exercise are relevant, including duration, intensity and frequency. Based on these factors, specific food categories can be defined, including food products, which give an increase of fluid/energy/nutrient availability per unit of time in order to optimise physical performance directly or indirectly by an improved rate of recovery from exercise.

The first and clearest difference in nutritional needs between individuals who are engaged in intense muscular exercise and the general population is related to energy expenditure.

The energy expenditure of a sedentary adult female/male amounts to approximately 8.5-12.0 MJ (1825 - 2580 kcal) per day. Physical activity by means of training or competition will increase the daily energy expenditure by 2 to 4 MJ (430 - 860 kcal) per hour of exercise, depending on physical

fitness and on duration, type and intensity of sport. For this reason, individuals with a substantial increase in their daily physical activity must adapt the energy intake by an increased food consumption to meet the energy needs according to the level of daily energy expenditure. This increased energy demand could be achieved by an increased intake of selected normal foods. Many athletic events are characterized by high exercise intensities. As a result, energy expenditure over a short period of time may be extremely high. For example, to run a marathon will take about 10-12 MJ (2150 - 2580 kcal). Depending on the time to finish, this may induce an energy expenditure of approximately 3.2 MJ (688 kcal)/h in a recreational athlete and 6.3 MJ (1355 kcal)/h in an elite athlete. A professional cycling race like the Tour de France, will cost an athlete about 27 MJ (5800 kcal)/day with extremes up to 40 MJ (8600 kcal)/day [136].

Compensating for such high energy expenditure by ingesting normal solid meals will pose a problem for any athlete involved in such competitions, since the digestion and absorption processes will be impaired during intensive physical activity. These problems are not restricted to competition days. During intensive training days, energy expenditure is also high. In such circumstances, athletes tend to ingest a large number of 'in between meals' up to 40% of the total energy compared to about 25% in the general population [159]. These 'in between meals' are often composed of energy rich snacks, but are often high in fat and low in protein and micronutrients. Such a diet would lead to a deficient nutrient intake if energy intake becomes low as is sometime observed in athletes such as gymnasts.

Specially adapted nutritious foods/fluids which are easily digestible and rapidly absorbable, may solve this problem.

In addition to the energy needs and the limited capacity and time to digest and metabolise foods, is the importance of the selection of fuels in the muscle. Metabolic capacity and power output depend on this selection. For maximal muscle performance the muscle cell depends almost entirely on carbohydrate (CHO) as a substrate. Therefore, the diet selection is not only a matter of energy but also a selection of the substrate sources, especially carbohydrate and fat.

At the other end of the energy expenditure scale it is seen that energy intake, especially in females athletes such as gymnasts and ballet dancers is often extremely low [159]. This can partly be explained by the urge to limit energy intake and to reduce body mass and particular fat mass. In addition eating disorders are frequently observed in these groups [135].

The low energy intakes in these circumstances may lead to a low intake of essential nutrients such as protein, iron, calcium, zinc, magnesium and vitamins. The CHO intake may not be sufficient to balance the CHO used in training. This aspect should receive special attention since many of these athletes are young and still in a period of growth and development.

Another essential difference between sedentary and very active individuals is the high rate of heat production and consequent sweat loss. It is therefore essential that, besides restoration of energy and nutrient loss, fluid and electrolyte replacement is secured. In terms of homeostasis and optimum performance, fluid (and electrolyte) replacement during exercise has the highest priority.

Finally, over the years a number of natural food ingredients have been identified and scientifically proven to be ergogenic. For example, supplementation of comparable modest levels of caffeine or high levels of creatine compared to the normal dietary intake, can benefit endurance and high intensity performance. At the same time it is not surprising that many faddish diets and exotic ingredients have come and gone.

It is against this background that the SCF examined the requirements of foods for particular nutritional uses to meet the demands of intense muscular effort, especially for sportspeople. The Committee has attempted to define a number of food categories and to evaluate the scientific evidence for the need and/or benefit of such food category for particular nutritional uses beyond the general accepted food habits and dietary guidelines. If possible, "composition and specifications" have been formulated.

The safety aspects of high level of intake of specific foods or food compounds are not taken into consideration in this report.

5. Categories of food products intended to meet the expenditure of intense muscular effort, especially for sportsmen.

Food products can be broadly classified on their intended functionality in relation to the physical exercise:

A Carbohydrate-rich energy food products
B Carbohydrate-electrolyte solutions
C Protein and protein components
D Supplements
D1 Essential nutrients:
- D1-1 Minerals
- D1-2 Trace elements
- D1-3 Vitamins
- D1-4 Essential fatty acids.

D2 Other food components
- D2-1 Caffeine
- D2-2 Creatine
- D2-3 Carnitine
- D2-4 Medium Chain Triglycerides (MCT)
- D2-5 Branched Chain Amino Acids (BCAA)

Each of these four categories are reviewed in greater detail below.

6. Category A Carbohydrate-rich energy food products

6.1. Background

The systematic study of the link between carbohydrate intake and exercise capacity began over sixty years ago. In a series of studies examining the link between diet and submaximal endurance cycling capacity Christensen and Hansen [35] showed that time to exhaustion is increased when a high carbohydrate diet is consumed for about three days before exercise. In contrast, time to exhaustion (endurance capacity) was shorter when the diet was low in carbohydrate, though adequate in fat and protein. These times to exhaustion were compared with the performance times of their subjects when they had consumed their normal mixed diets in the days before exercise.

Thirty years later the explanation for the improvement in exercise capacity was provided by several studies, which used a percutaneous, needle biopsy technique to obtain small samples of muscle before, during and after exercise [17, 16]. Bergstrom and colleagues showed that fatigue during prolonged heavy submaximal exercise was closely associated with very low muscle glycogen concentrations. Those subjects who began submaximal exercise with the largest muscle glycogen stores tended to cycle longer than those with smaller stores. Therefore, dietary and exercise manipulations were designed to increase muscle glycogen stores before prolonged exercise in order to improve endurance capacity [2, 140]. Most studies using dietary carbohydrate loading in the days before prolonged submaximal exercise report improvements in endurance capacity during subsequent exercise [4, 22, 63, 115, and 134]. However, some studies report no improvement in performance following carbohydrate loading [99, 140].

The benefits of carbohydrate loading are most clearly demonstrated during prolonged submaximal exercise where the endpoint is fatigue. However, when the exercise demands that the participants complete a fixed distance in the shortest possible time (e.g. endurance races) then there are factors, other than muscle glycogen stores, which dictate the performance of the athlete. For example, running speeds are largely dictated by the maximum rate of oxygen consumption and training status of participants in endurance races. During heavy exercise lasting about an hour, the normal high muscle glycogen stores of athletes are usually sufficient to cover the demands of muscle metabolism. This is illustrated in the study reported by Sherman et al [140] where well-trained runners completed 20.9-Km races after dietary carbohydrate loading and after eating their normal mixed diets. There were no differences in the times taken to complete the distance (approximately 83-min) even though there were significant differences in pre-exercise muscle glycogen stores. Furthermore, it is clear from this latter study and an earlier study [81] that high pre-exercise muscle glycogen stores do not

enable athletes to run faster in the early part of a race, although, they do allow them to maintain their chosen running speed for longer. It is this capacity to maintain their race pace which leads to improved performance times. Thus, carbohydrate loading improves performance only when the duration of races is such that they make large demands on muscle glycogen stores. This is also true for prolonged heavy intermittent exercise as seen in sports as such as soccer, rugby and hockey [115, 179].

Endurance trained athletes use less muscle glycogen than less well-trained individuals during submaximal exercise of the same absolute intensity. The greater aerobic capacity of the skeletal muscles of athletes allows them to use more fat for energy metabolism and so they use less glycogen. Nevertheless athletes need a high carbohydrate diet to support their heavy daily training because muscle glycogen is the main fuel for prolonged high intensity exercise.
Health professionals recommend that we should eat a diet, which provides at least 50% of our daily energy intake in the form of carbohydrates. A daily energy intake of approximately 10.5 MJ (2500 kcal) would therefore provide about 310 g of carbohydrate or 4.5 g/kgbw for a 70-kg man. This should provide sufficient carbohydrate to cover an active lifestyle and many recreational activities which do not produce exhaustion. However, a carbohydrate intake of about 5 to 6 g/kgbw is required to support daily exercise which is of moderate to high intensity and lasts no more than an hour. This amount of carbohydrate can be achieved simply by changing the composition of the diet to include more carbohydrate containing foods. Nevertheless, during activities in which fatigue limits performance, dietary carbohydrate loading will be of benefit to recreationally active people as well as to athletes preparing for competition. During this preparation period, a daily carbohydrate-intake of approximately 70% of energy intake has been recommended [49]. Therefore, in order to achieve high muscle and liver glycogen stores before exercise, it is essential to consume foods, which can collectively provide the necessary amount of carbohydrate.
The following is a brief summary of the nutritional strategies adopted to prepare for participation in and recovery from sport and exercise.

6.2. Pre-Exercise Meals

The current method of 'carbohydrate-loading' during the week prior to competition is to gradually reduce the volume of training throughout the week and to increase the carbohydrate intake to about 600 g/day during the last four days before the event [140]. Muscle glycogen concentration is increased above normal resting values as a consequence of this dietary preparation for competition. However, the recommended amount of carbohydrate may not be appropriate for female athletes because, for many, it would be equivalent to their daily energy intake (approximately 10 MJ (2400 kcal). Therefore, it is more appropriate to prescribe daily carbohydrate intake in terms of g/kgbw. Expressed in this way the recommendation for

carbohydrate loading is a daily intake of 9 to 10g/kgbw of carbohydrate during the days immediately preceding competition.

A wide range of carbohydrate-containing foods appear to be equally effective in increasing muscle glycogen concentrations following carbohydrate loading [132] and increasing endurance running capacity [4, 22, 63, 115, 134]. However, one study suggests that there are advantages to eating low glycaemic index carbohydrate foods (Table 1) before exercise because they provide a slow release of glucose for muscle metabolism [150]. Logical as this advice may sound, the performance benefits of pre-exercise meals containing low glycaemic index carbohydrates have not been confirmed in more recent cycling and running studies [55, 175].

Table 1 **EXAMPLES OF MEAN GLYCAEMIC INDICES OF COMMON FOODS**

Breads and Grains		**Fruits**			
Rice, instant	91	Watermelon	72	Milk, skim	32
Wheat bread, white	70	Pineapple	66	Milk, full fat	27
Bread, whole wheat	69	Raisins	64		
Cornmeal	68	Banana	53	**Snacks**	
Rice, white	56	Grapes	52	Rice cakes	82
Rice, brown	55	Orange	43	Jelly beans	80
Mixed grain bread	45	Pear	36	Corn chips	73
Spaghetti, white	41	Apple	36	Candy bar	68
Spaghetti, whole wheat	37			Wheat crackers	67
Rye	34	**Starchy Vegetables**		Popcorn	55
Barley	25	Potatoes, baked	83	Oatmeal cookies	55
		Potatoes, instant	83	Potato chips	54
Breakfast cereals		Potatoes, mashed	73	Chocolate	49
Corn Flakes	84	Carrots	71	Banana cake	47
Rice Krispies	82	Sweet potatoes	54	Peanuts	14
Grape Nuts Flakes	80	Green peas	48		
Shredded wheat	69			**Sugars**	
Grape Nuts	67	**Legumes**		Honey	73
Oatmeal	61	Baked beans	48	Sucrose	65
Porridge	61	Chick peas	33	Lactose	46
Muesli	52	Butter beans	31	Fructose	23
All Bran	42	Lentils	29		
		Kidney beans	27	**Beverages**	
				Sports drinks	95
		Dairy		Soft drinks	68
		Ice cream	61	Orange juice	57
		Yoghurt, low fat sweetened	33	Apple juice	41

Foods listed from highest to lowest glycaemic index within category. Glycaemic index was calculated using glucose as the reference with GI of 100. Modified from Foster-Powell and Brand Miller [59].

COMMENT:

The given glycaemic indices are not fixed values. Mostly they represent a range that is based on production process and preparation of the food. For example: wheat bread: white flour; 5 studies, mean±SE:70±0; whole meal flour 12 studies mean±SE:69±2.

The pre-exercise meal, eaten no later than 2 to 3 hours before exercise, should be easy to digest and high in carbohydrates. Adopting this dietary recommendation improves endurance capacity during cycling and running [36, 181] when compared with fasting before exercise.

6.3. Food intended to be consumed during exercise

Eating during exercise is a practical option in only a few sports. For example, long distance cyclists and canoeists, as well as triathletes, eat easy to digest carbohydrate snacks during races. High-energy bars and confectionery products tend to be the snacks of choice because they are energy dense and easy to carry. Carbohydrate-electrolyte solutions are a convenient way of obtaining fluids, to off-set dehydration, and fuels to delay the onset of fatigue [106, 153] (see section on carbohydrate-electrolyte solutions).

6.4. Food intended to be consumed after exercise

Although much attention has been paid to nutritional preparation for exercise, nutritional intervention during recovery has received much less attention. In order for athletes to train or compete daily they must recover quickly. Replacing muscle and liver glycogen stores as rapidly as possible is essential for successful recovery. Failure to restock these carbohydrate stores will prevent athletes from completing prolonged periods of heavy exercise [43]. Glycogen resynthesis is most rapid during the first few hours after exercise [125]. Therefore, consuming carbohydrates immediately after exercise results in a greater rate of glycogen resynthesis than occurs when intake of carbohydrate is delayed [74, 83]. The provision of carbohydrates, as a substrate for glycogen synthesis, also stimulates a release of insulin, which enhances the uptake of glucose by muscle. The increased permeability of muscle to glucose is a post-exercise phenomenon that is a consequence of an activation of glucose transporter proteins (GLUT 4) [76]. Therefore, it is not surprising that the most effective carbohydrate foods for rapid glycogen resynthesis are those which have a high glycaemic index because not only will they provide glucose but they will also stimulate a large increase in plasma insulin concentration [28, 85]. The post-exercise presence of insulin complements the action of exercise on GLUT 4 transporter proteins by increasing the availability of these transporter proteins during the recovery period [76].

Consuming approximately 1 g per kgbw of carbohydrate immediately after exercise and every 2 hours up to 6 hours of recovery increases the rate of muscle glycogen synthesis (50 %) above that which would be achieved without consuming carbohydrate [75]. A carbohydrate intake of very much more than 1 g per kgbw (e.g. 2 to 3 g) does not appear to produce a further increase in the rate of glycogen resynthesis in the first hours after exercise. However, there is some evidence to suggest that adding protein to the recommended amount of carbohydrate may further increase the rate of glycogen resynthesis [182]. The explanation for this increased resynthesis rate is the higher flux of carbohydrate into the muscle cell caused by

the increased concentration of circulating insulin. Also single amino acids can raise the insulin concentration considerably [58]. Recent studies have shown that a combination of carbohydrate and proteins (hydrolysates and/or amino acids) maximize post-exercise muscle glycogen synthesis compared to carbohydrate supplementation alone [162].

The concentration of muscle glycogen restored during recovery after exercise is proportional to the total amount of carbohydrate consumed. Figure 1 shows the relationship between the amount of carbohydrate consumed during 24 hours recovery from exercise and the increases in muscle glycogen concentrations.

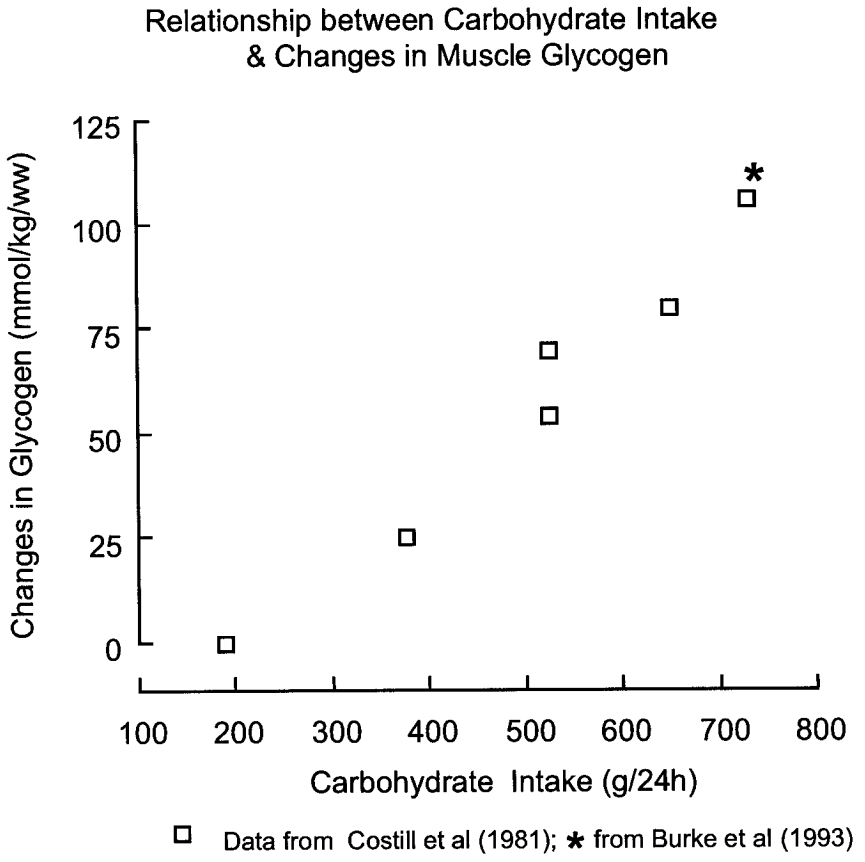

Figure 1

Result from 24-h post-exercise recovery studies in changes in muscle glycogen and carbohydrate intake.

Having followed the recommendation to restock muscle glycogen stores, the obvious question is whether or not performance will also be restored. The available research shows that a high carbohydrate diet during recovery does restore endurance performance during subsequent exercise. For example, raising the carbohydrate intake from 5 g/kgbw to 9 - 10g/kgbw during 24 hour recovery allowed endurance runners to match their 90 minute training run of the previous day. However, on another occasion, when they had their normal intake of carbohydrate, along with additional protein and fat to match their energy intake on the carbohydrate recovery diet, they were unable to complete the 90-minute run [53]. A high carbohydrate diet during recovery from prolonged intermittent exercise has also been reported to improve subsequent endurance performance [4, 115]. These studies clearly show that it is the additional carbohydrate in the recovery diet which is responsible for the restoration of performance rather than the intake of energy in the form of protein or fat.

6.5. Composition and specifications for carbohydrate- rich energy food products

The product should supply at least 75 % carbohydrate as a main source of energy (Energy %). If it is a drink, the carbohydrate concentration should exceed 10 % of weight by volume. At least 75 % of energy should be derived from metabolisable carbohydrates. Metabolisable carbohydrates such as glucose, glucose polymers, sucrose and carbohydrates with similar properties are those types of carbohydrates with a high glycaemic index.

In addition, these products may contain at least 0,05 mg vitamin B_1 (thiamine) per 100 kcal provided by carbohydrates (at least 0,2-mg vitamin B_1 (thiamine) per 100 g of carbohydrates).

7. Category B Carbohydrate-electrolyte solutions (C.E.S.)

7.1. Background

The aim of the athlete who ingests drinks before, during or after training or competition is to improve performance, and this can be achieved by minimising the impact of the factors that cause fatigue and impair the performance of skilled tasks. The two factors that have been considered to contribute most to the onset of fatigue in exercise are the depletion of the body's carbohydrate reserves and the onset of dehydration resulting from the loss of water and electrolytes in sweat [102]. There are good reasons for taking drinks containing added carbohydrates and electrolytes.

Commercially formulated sports drinks are intended to serve a variety of purposes, including supply of substrate, prevention of dehydration and promotion of post-exercise recovery.

7.2. Formulation of sports drinks

The major components of the sports drink that can be manipulated to alter its functional properties are shown in table 2.

Table 2

Variables that can be manipulated to alter the functional characteristics of a sports drink
- Carbohydrate content: concentration and type
- Osmolality
- Electrolyte composition and concentration
- Other ingredients (such as caffeine, see category D2)

7.3. Carbohydrate content: concentration and type

Many studies have shown that the ingestion of glucose during prolonged intense exercise can prevent the development of hypoglycaemia by maintaining or raising the circulating glucose concentration. Beneficial effects of carbohydrate ingestion are seen during cycling as well as during running. This ergogenic effect may be related to a sparing of the body's limited muscle glycogen stores by the oxidation of the ingested carbohydrate, but the primary benefit of ingested carbohydrate is probably its role in supplementing the endogenous stores in the later stages of exercise [44]. It is clear from tracer studies that a substantial part of the carbohydrate ingested during exercise is available for oxidation, but there appears to be an upper limit of about 1 gram per minute to the rate at which ingested carbohydrate can be oxidised, even when much larger amounts are ingested [168].

As well as providing an energy substrate for the muscles, addition of carbohydrate to drinks can promote water absorption in the small intestine. It is sometimes difficult to separate the effects of water replacement from those of substrate and electrolyte replacement when CHO-electrolyte solutions are ingested, but Below et al [8] have shown that ingestion of carbohydrate and water had separate and additive effects on exercise performance. Most reviews of the available literature have come to the same conclusion [92, 113, 102, 106]. Most of the common types of carbohydrates such as glucose, sucrose and oligosaccharides are effective in maintaining the blood glucose concentration and in improving endurance capacity. Substitution of glucose polymers for glucose will allow an increased carbohydrate content without an increased osmolality, and may also have taste advantages, but the available evidence suggests that the use of glucose polymers rather than free glucose does not alter the blood glucose response or the effect on exercise performance, and similar effects are seen with the feeding of sucrose or mixtures of sugars. Some studies have suggested that long chain glucose polymer solutions are more readily used by the muscles during exercise than are glucose or fructose solutions, but others have found no difference in the oxidation rates of ingested glucose or glucose polymer. Massicote et al [101] also found that ingested fructose was less oxidized during exercise than glucose or glucose polymers during exercise.
Mixtures of glucose and fructose in equal amounts seem to have some advantages: when ingested in combination there is an increased total exogenous carbohydrate oxidation. Fructose in high concentrations is generally best avoided on account of the risk of gastrointestinal upset. There may be benefits in including a number of different carbohydrates, including free glucose, sucrose and maltodextrin: this has taste implications, which may influence the amount consumed, and, by limiting the osmolality and providing a number of transportable solutes, may maximize the rate of sugar and water absorption in the small intestine [141].

The optimum concentration of carbohydrate to be added to drinks will depend on individual circumstances. High carbohydrate concentrations will delay gastric emptying, thus reducing the amount of fluid that is available for absorption, but will increase the rate of carbohydrate delivery. If the concentration is high enough to result in a markedly hypertonic solution, net secretion of water into the intestine will result, and this will actually increase the danger of dehydration. High carbohydrate concentrations (>10%) may also result in gastro-intestinal disturbances. Where the primary need is to supply an energy source during exercise, increasing the carbohydrate content of drinks will increase the delivery of carbohydrate to the site of absorption in the small intestine. Beyond a certain limit, however, simply increasing carbohydrate intake will not continue to increase the rate of oxidation of exogenous carbohydrate [168]. Dilute glucose-electrolyte solutions may also be as effective, or even more effective, in improving performance as more concentrated solutions and adding as little as 90-mmol/l (16-g/l) glucose may improve endurance performance [105].

7.4. Osmolality

It has become common to refer to carbohydrate-electrolyte sports drinks as isotonic drinks, as though the tonicity was their most important characteristic. The osmolality of ingested fluids is important as this can influence both the rates of gastric emptying and of intestinal water flux: both of these processes together will determine the effectiveness of rehydration fluids at delivering water for rehydration. An increasing osmolality of the gastric contents will tend to delay emptying, and increasing the carbohydrate or electrolyte content of sports drinks will generally result in an increased osmolality. The composition of the drinks and the nature of the solutes is, however, of greater importance than the osmolality itself [102].

Although osmolality is identified as an important factor influencing the rate of gastric emptying of liquid meals, there seems to be rather little effect of variations in the concentration of sodium or potassium on the emptying rate, even when this substantially changes the test meal osmolality [130]. The effect of increasing osmolality is most consistently observed when nutrient-containing solutions are examined, and the most significant factor influencing the rate of gastric emptying is the energy density. Vist and Maughan [164] have shown that there is an acceleration of emptying when glucose polymer solutions are substituted for free glucose solutions with the same energy density: at low (about 40 g/l) concentrations, this effect is small, but it becomes appreciable at higher (180 g/l) concentrations; where the osmolality is the same (as in the 40 g/l glucose solution and 180 g/l polymer solution), the energy density is shown to be of far greater significance in determining the rate of gastric emptying. This effect may be important when large amounts of energy must be replaced after exercise, but is unlikely to be a major factor during exercise where more dilute drinks are taken. Water absorption occurs largely in the proximal segment of the small intestine, and, although water movement is itself a passive process driven by local osmotic gradients, is closely linked to the active transport of solute. Osmolality plays a key role in the flux of water across the upper part of the small intestine. Net flux is determined largely by the osmotic gradient between the luminal contents and intracellular fluid of the cells lining the intestine. Absorption of glucose is an active, energy-consuming process linked to the transport of sodium. The rate of glucose uptake is dependent on the luminal concentrations of glucose and sodium, and dilute glucose-electrolyte solutions with an osmolality, which is slightly hypotonic with respect to plasma, will maximize the rate of water uptake. Solutions with a very high glucose concentration will not necessarily promote an increased glucose uptake relative to more dilute solutions, but, because of their high osmolality, will cause a net movement of fluid into the intestinal lumen [62]. This results in an effective loss of body water and will exacerbate any pre-existing dehydration. Other sugars, such as sucrose or glucose polymers can be substituted for glucose without impairing glucose or water uptake, and may help by increasing the total transportable substrate without increasing osmolality. In contrast, iso-energetic solutions of fructose and glucose are isosmotic, and the absorption of fructose is not an active process in man: it is absorbed less rapidly than glucose and promotes less water uptake.

The use of different sugars which are absorbed by different mechanisms and which might thus promote increased water uptake is supported by recent evidence from an intestinal perfusion study [141]. Although most of the popular sports drinks are formulated to have as close to that of body fluids [102] and are promoted as isotonic drinks, there is good evidence that hypotonic solutions are more effective when rapid rehydration is desired. Although it is argued that a higher osmolality is inevitable when adequate amounts of carbohydrate are to be included in sports drinks, the optimum amount of carbohydrate necessary to improve exercise performance has not been clearly established.

7.5. Electrolyte composition and concentration

The only electrolyte added to drinks consumed during exercise that is known to confer physiological benefit is sodium. Sodium will stimulate carbohydrate and water uptake in the small intestine and will help to maintain extracellular fluid volume. Most soft drinks of the cola or lemonade variety contain virtually no sodium (1-2 mmol/l); sports drinks commonly contain about 10-30 mmol/l; oral rehydration solutions intended for use in the treatment of diarrhoea-induced dehydration, which may be fatal, have higher sodium concentrations, in the range 30-90 mmol/l. If exercise duration is likely to exceed 3-4 h, addition of sodium helps avoid the danger of hyponatraemia, which occurs when excessive volumes of low-sodium drinks are taken. Supplementation with sodium salts may be required in extremely prolonged events where large sweat losses can be expected and where it is possible to consume large volumes of fluid.

Restoration of fluid and electrolyte balance after exercise is an important part of the recovery process, especially when a second exercise session must be performed after a short time interval. Urine output in the few hours after exercise when volume replacement is undertaken is inversely proportional to the sodium content of the ingested fluid, with an almost linear relationship between net sodium balance and net water balance [103]. Only when the sodium content exceeded 50 mmol/l were the subjects in positive sodium balance, and only then did they remain in positive fluid balance throughout the recovery period. Shirreffs et al. [142] showed that even drinking large volumes (twice the sweat loss) did not allow subjects to remain in positive fluid balance for more than 2 h when the sodium content of the drinks was low (20 mmol/l): increasing the sodium content to 60 mmol/l allowed subjects to remain well hydrated when volumes equal to 1.5 times or twice the sweat loss were ingested.

It has been speculated that inclusion of potassium, the major cation in the intracellular space, would enhance the replacement of intracellular water after exercise and thus promote rehydration [114]. Potassium is normally present in commercial sports drinks in concentrations similar to those in plasma and in sweat, but there is little evidence to support its inclusion. Although there is some loss of potassium in sweat (about 3-7 mmol/l), an increase in the circulating potassium concentration is the normal response to exercise: increasing this further by ingestion of potassium does not seem useful. A similar situation applies with magnesium replacement, and in spite of the commonly held

belief that exercise-induced cramp is associated with a falling plasma magnesium concentration, there is little or no experimental evidence to substantiate this belief. A slight decrease in the plasma magnesium concentration is generally observed during exercise, but this seems to be the result of a redistribution of the body magnesium stores, and there is no good scientific reason for its addition to sports drinks [106].

7.6. Composition and specification for carbohydrate-electrolyte solutions

The drink should supply carbohydrate as the major energy source and should be effective in maintaining or restoring hydration status.

To achieve this, these beverages should contain not less than 80-kcal/1000 ml and not more than 350 kcal/1000 ml. At least 75 % of the energy should be derived from metabolisable carbohydrates characterised by a high glycaemic index. Examples are glucose, glucose polymers and sucrose. In addition these beverages should contain at least 20 mmol/l (460 mg/l) of sodium (as Na^+) and not more than 50 mmol/l (1150 mg/l) of sodium (as Na^+). They may be formulated to cover a range of osmolalities between 200 and 330 mOsml/kg water. Beverages with an osmolality of 300-m Osm +/- 10 % range (270 - 330 mOsm/kg water) may be designated as isotonic.

8. Category C Protein and protein components

8.1. Background

There is a long history from Ancient Greece till modern times that athletes and their trainers believe that the daily dietary protein requirements of subjects participating in endurance or strength training programs are increased. The rationale for the increased intake differs between endurance and strength training athletes. In the case of endurance exercise it is assumed that the rate of protein oxidation increases during exercise and that this should be compensated in the recovery period following exercise. In the case of strength training it is assumed that muscle hypertrophy and increases in strength can only be maximal when the dietary protein intake is high. However, among scientists this is an issue of continued discussion, as it is not clear which underlying physiological processes lead to an increased metabolic need for protein. In this respect there is a major difference compared with the effect of an increase in carbohydrate intake, which has been shown to improve endurance performance both when taken acutely and when the carbohydrate content of the diet is increased.

Concern has been raised relative to the possible long-term health-hazards of protein intakes as high as 2 - 4 g protein/kgbw, such as an impaired kidney function and/or negative calcium balance. However, scientific evidence and consensus is lacking on these issues.

8.2. Protein metabolism in endurance athletes

In the 1840's the German physiologist Von Liebig hypothesized that muscle protein was the main fuel used to achieve muscular contraction [165]. Von Liebig's ideas have lead to the 19th and 20th century belief that industrial workers exposed daily to heavy physical labour and a high energy expenditure have an increased protein requirement and, therefore, should eat large steaks and have protein rich nutrition. In the late 19th century the hypothesis that muscle protein was the main fuel for physical exercise was shown to be false as controlled nitrogen balance studies as early as 1866 [56, 32] failed to show a substantial increase in nitrogen losses during and following exercise. Also in recent literature most of the controlled nitrogen balance studies show that trained athletes accustomed to a high energy expenditure (e.g. running = 125 km per week [144]) or several days of cycling with energy expenditures of 25 MJ per day [24] can maintain a positive or zero N-balance on diets containing 1.0-1.4 g protein per kgbw per day [65, 107, 24, 61, 144, 96]. Only when subjects are fasted overnight or for longer periods [94] may exercise lead to increased N-losses.

In summary, N-balance studies suggest that there is a modest increase and therefore the recommended daily intake has been increased from 1.2 to 1.4 g per kgbw per day in elite endurance athletes. A diet containing 10-12%En protein contains enough protein to meet this modest increase in the protein requirement as the daily energy expenditure of elite endurance athletes can be as much as two to three-fold higher than that of normal subjects.

With the introduction of stable isotope amino acids, new techniques became available to investigate protein metabolism and its components (protein oxidation, protein synthesis and protein degradation) during exercise. Early studies [180] using with a leucine tracer suggested that protein oxidation was increased and that whole body protein synthesis (indirectly calculated as protein flux minus protein oxidation) was decreased during exercise in man. However, direct estimates of muscle protein synthesis did not show a difference between rest and exercise [29]. Whole body urea production, another measure of protein oxidation, was similar at rest, during 3 h of treadmill running at 40% VO_{2max} and during 1 h of exercise at 70% VO_{2max} [30]. Recently Wagenmakers et al. [169] performed a study with 3 tracers in highly trained subjects ingesting carbohydrates (as athletes do in sports practice) during 6 h of exercise (cycling-running-cycling) at 50% VO_{2max}. In agreement with Wolfe [180] leucine as tracer suggested that protein oxidation was 2- to 3-fold higher during exercise. However, the other tracers failed to confirm this. This indicates that the metabolic needs of endurance athletes for dietary protein do not seem to be substantially increased.

Recent studies [182, 160, 162] have shown that the combined ingestion of glucose and protein increases glycogen re-synthesis and plasma insulin levels in the post-exercise period (see also section Carbohydrate-rich energy food products). Calculations based on glycogen resynthesis rates measured in the laboratory show that it takes 16-20 h before the glycogen stores are fully replenished after exercise and that co-ingestion of protein may accelerate glycogen re-synthesis by 4-8 h [170]. This effect of protein co-ingestion could explain an increased metabolic need for protein of endurance athletes and could provide a metabolic rationale for an increment of the Recommended Daily Allowances. More research is needed to investigate how much additional protein is needed to reach this effect, and whether the increased protein intake is required in all the post-exercise meals or only in a limited post-exercise time frame. In the latter case it could well be true that the metabolic need of endurance athletes for protein is only marginally increased, but that more attention should be given to the timing of the intake of the extra protein.

8.3. Protein metabolism in body builders and strength athletes

Tarnopolsky et al. [144] reduced the protein intake of 6 male elite body builders from the habitual intake of 2.77 g per kgbw per day to 1.05 g per kgbw per day (isoenergetic diets) and observed that they were able to maintain a zero or positive nitrogen balance on this low protein intake. Tarnopolsky et al. [146] investigated the effect of a low, moderate and high protein intake on N-balance in a group of young male strength athletes (training for about 2 months) and observed that they reached a zero N-balance on 1.4 g protein per kgbw per day. A similar value was observed by Lemon et al. [95] in novice bodybuilders starting an intensive body building program. A further increase of the protein intake to 2.62 g per kgbw per day in the latter study did not increase the strength and muscle mass gain during a one-month training

program. Based on these studies it can be concluded that young strength athletes and novice body builders (with a rapid muscle mass gain and strength gain) reach zero N-balance on about 1.4-1.5 g per kgbw per day. In elite body builders, training for several years, the protein requirements are assumed to be only marginally higher than in sedentary subjects [147].

Acute and long-term increases in the protein content of the diet do not by definition lead to increases in muscle protein mass or whole body protein mass when the initial protein content exceeds the dietary requirement [129]. There are several physiological reasons for this. The main reason is that most of the enzymes involved in the protein synthetic machinery of the cells and organs of the body have a low K_m. They operate at maximal velocity when the intracellular amino acid concentration is between 10 and 30 μM and basal levels of amino acids generally are much higher. Therefore, an acute increase in the plasma or intracellular concentration of the amino acids following the ingestion of a meal with a high protein content does not lead to a substrate activated increase in the rate of protein synthesis [129]. An increase in the protein synthesis rate will occur after ingestion of a protein containing mixed meal as a consequence of the insulinotropic effect of protein/carbohydrate ingestion [129, 58]. The K_m for the oxidative enzymes (e.g. urea cycle enzymes, dehydrogenases) is much higher. This implies that the rate of amino acid oxidation and urea production increase rapidly following ingestion of a meal with a high protein content. The main effect of an acute increase in the protein content of the diet, therefore, is increased amino acid oxidation when the protein intake exceeds protein requirement. Tarnopolsky et al [146] observed a significant (+30%) increase in whole body protein synthesis (measured with ^{13}C-leucine as tracer) in young male strength athletes when the protein intake was increased from 0.86 to 1.40 g per kgbw per day. No further increase was observed when the protein intake was further increased to 2.4 g per kgbw per day. In a group of sedentary controls maximal whole body protein synthesis rates were observed already at the lowest protein intake. This again indicates that the protein requirement in strength athletes is slightly increased to the recommended protein intake of about 1.4-g per kgbw per day.

Protein hydrolysates and balanced mixtures of free amino acids are more rapidly absorbed than intact proteins and lead to higher increases in plasma amino acid concentration than intact proteins. Due to the difference in enzyme kinetics indicated above, the hydrolysates and free amino acids are more rapidly oxidized and less efficiently used for protein synthesis [40]. Therefore the effectiveness of protein hydrolysates as means to increase muscle protein synthesis in athletes is not supported by scientific evidence. When the increased protein content of the diet is maintained for several days, then the concentration of the enzymes involved in amino acid oxidation will start to increase too [129]. Maximal concentrations are seen only after 1 to 2 weeks. This induction of the oxidative enzymes induced by a high protein diet also leads to more protein being oxidized more rapidly on a high protein diet. This induction in part also explains the lower plasma amino acid levels and increased amino acid oxidation rates that are seen in the overnight fasting period in subjects on a high protein diet [121].

Leucine oxidation has been reported to be similar at rest and during and following resistance-type exercise [145] indicating that increased amino acid oxidation does not lead to an increased protein requirement. However, it has been shown clearly that muscle protein synthesis and protein degradation increase by 50-100% in the first hours after resistance-type exercise [34, 98, 19, 124] in previously untrained young male subjects. The increase in muscle protein synthesis persists for 24 hours and returns to baseline after 36 hours [98, 124] This increase in protein turnover most probably is essential for the increase in myofibrillar protein content and remodelling of the muscle structure that is following heavy resistance exercise [97]. The increase in muscle protein synthesis following resistance exercise is also seen in 62- to 75-yr-old men and women [176]. In these subjects an increase of the protein content of the post-exercise meal from 7 to 14 and to 21 % En did not further increase the protein synthesis rate in muscle.

8.4. Composition and specifications for protein and protein components

* General considerations
  - The Committee recommends that the protein source used for these products should have a minimal protein quality (NPU) level
  - Based on the concept that the requirement for vitamine B_6 is closely related to the protein intake the Committee recommends additional supplementation of vitamine B_6 per gram of protein [41].

* Protein concentrates
  The product should supply at least 70 % of the dry matter as protein with at least a NPU quality 70 % or higher.

* Protein enriched foods
  Foods presented as protein enriched should contain at least 25 % of the total energy as protein with protein quality (NPU) of 70 % or higher.

* Amino acids addition is allowed for the purpose of improving the nutritional value of the protein, in the proportion necessary of that purpose.

* In addition, these products may contain vitamin B_6. If added, it should contain at least 0.02-mg vitamin B_6 per gram of protein.

9. Category D Supplements

9.1. Category 1 Essential nutrients

9.1.1. Background

Recommended dietary allowance for essential nutrients in healthy adults are published by various national nutrition boards and are also given in the reports of the Scientific Committee for Food (nutrient and energy intakes for the European Community) [41] (Tab.1). They do not distinguish between recommended dietary allowances for normal healthy or physically active people. Nevertheless, in case of intensive physical exercise and regularly performed training there are some problems and peculiarities in practice and reactions to sports which may influence the balance and requirements for some essential nutrients [11, 26, 178].

For some vitamins and minerals there are potential hazards from the intake of high levels of these nutrients, especially among athletes where supplementation with high doses of vitamins and minerals is very popular. The upper safe levels of vitamin- and mineral intake are at present under consideration of the SCF and will not be covered in this report.

Other non-nutrients are considered on the basis of their ergogenic properties. For the evaluation of the sports-specific requirements of micronutrients some general points should be taken into account.

9.1.2. General considerations

9.1.2.1. Energy related nutrient density

It is a frequently asked but an open question whether there is a definite, linear correlation between total energy output and the requirement for nutrients. Diet balances have not supported the common assumption that there is a generally increased requirement for essential nutrients in athletes induced by their physical exercise or data from controlled studies. Even in the case of energy related micronutrients such as vitamin B_1 (thiamine) which functions as a co-enzyme in carbohydrate metabolism no conclusive data are available. The recent US RDA's concluded that those who are engaged in physical demanding occupations or who spend much time training for active sports may require additional vitamin B_1 [60]. This conclusion reflects the uncertainty about the energy related nutrient requirements. The hypothesis of a possible exercise-induced over-proportional need for a special group of essential micronutrients is nowadays obsolete. With regard to a causative positive correlation between energy output produced by physical exercise and additional nutritional requirements for micronutrients it is accepted that these additional requirements are sufficiently covered by an energy-related, additional uptake of well-balanced food. However, under conditions of endurance exercise and training there may be an increased consumption of carbohydrate-rich diets as sports drinks or snacks showing no optimal nutritional density. To compensate this possible

disadvantage in sports practice athletes often take nutrient supplements to adjust the difference between recommended and actual nutrient density [26, 178].

9.1.2.2. Sports related factors

In combination with intensive physical exercise and regular training, some sports-specific endogenous and exogenous factors can influence the balance and therefore the requirements for essential and micro-nutrients during and after exercise, e.g. increased loss of nutrients in urine and sweat, increased cellular uptake and modulated distribution of nutrients in the body compartments, loss of nutrients by haemolysis and intestinal bleeding or decreased absorption of nutrients by the circulation-deficient intestinal tract [15, 25, 112]. Without the possibility of measuring nutrient balances or even nutrient deficiencies athletes often take nutrient supplements to compensate this possible sports-related disadvantage.

In weight category sports or in sports with a competitional benefit for underweight subjects, athletes are tempted to reduce their body weight by chronic energy restrictions. In combination with energy restrictions and the sports-related factors mentioned above, female athletes represent a particularly high-risk population group for deficiencies of essential nutrients. Athletes showing a total energy intake below 10.5 MJ (2.500 kcal)/day for males and below 8.4 MJ (2.000 kcal)/day for females, respectively, may not be able to reach their daily nutrient requirements during periods of regular training [159].

9.1.2.3. Exercise related health risks

Beside those conditions which are directly related to physical exercise and training it has to be considered that under special circumstances (e.g. started training, irregular training, inadequate training) athletes may be defined as a population group at an increased health risk. Therefore, new aspects in sports nutrition are added with regard to health promotion instead of athletic performance. Food composition and essential nutrients may influence the following known sports-related negative side effects also:

** Upper respiratory tract infections (URTI)*

Athletes, in particular endurance athletes with high training volumes, show an increased risk for URTI [57, 116]. Experience suggests that athletes may benefit from a diet rich in immune-stimulating Nutrients [12, 13, 119, 139].

** Exercise-induced inflammation*

Athletes and in particular endurance athletes again, also show an increased risk for exercise-induced tissue injuries and overuse syndromes [23, 131]. Since these tissue injuries are in general accompanied not by muscle and tissue soreness alone but also by an acute inflammatory response [27, 117], it has been discussed whether the amount of this exercise-induced inflammation can be reduced by food quality or selected nutritional supplements [119].

** Oxidative stress*

A new aspect of a health related consideration of sports is the ongoing discussion about increased oxygen uptake during physical exercise: Maximum physical work load can result in an increase of free radical production and the subsequent increase of oxidative stress [82]. Athletes may belong to the population group with an increased risk for oxidative stress and has been suggested that athletes should optimise their body pool of antioxidants by an increased intake. However, at present there is no scientific agreement on the question of whether a sufficient quantity of antioxidants, in particular of vitamin E, vitamin C and ß-carotene can be supplied by daily food intake alone [18, 91]. It is also unclear whether this increase is necessary due to an increased activity of endogenous free radical scavengers enzymes during training.

9.2. Minerals (K, Mg, Ca)

Key minerals involved in muscular functions include K, Ca and Mg, but there are no objective data which attest that dietary intake of these minerals is insufficient in athletes as compared with the recommended dietary intake. As mentioned above, it is likely that athletes consume sufficient amounts of minerals by increasing their energy intake during training periods. There may be a deficiency of minerals, however, in athletes under conditions of chronic energy restriction [11, 26, 159].

The potassium (K) content of the body is approximately 2-g/kg-body weight. K is the principal cation occurring in cell water and most of total body K mass is found in skeletal muscle, partly coupled to by glycogen storage; only 0.4% of the total body K mass is found in the plasma compartment. The working muscle loses potassium during contractions. Consequently plasma K concentration is increased during exercise; the amount of the K increase is directly correlated to the intensity of the exercise performed. In contrast to conditions during exercise, in rest plasma K concentrations of trained individuals are often lower (4.0-4.2 mmol/l) as compared to concentrations in untrained subjects [9]. This phenomenon can be explained by the training-induced adaptation of the cellular potassium uptake and a higher activity of the specific transport system, the Na-K-ATP-ase [39]. In that way moderately reduced plasma K concentrations (4.0-4.2 mmol/l) during resting conditions can not be used as indices of impaired K balance in athletes.

The magnesium (Mg) content of the body is 270-400 mg/kg body weight. About 95% of this mass is found within the cells, about 70% is in the skeleton and only a small extracellular fraction (1.3-%) is only metabolically available. Low plasma magnesium concentrations in athletes have been found at resting conditions as well as after exercise. These findings should not be interpreted as a symptom of Mg deficiency and may be explained by an exercise-induced redistribution of Mg. However, objective data about Mg deficiency in healthy athletes or about significant benefits of Mg supplementation from controlled intervention studies are not available.

The calcium (Ca) content of the body amounted to about 1200 g (16 g/kg body weight), approximately 99% is found in the skeleton, the small plasma fraction (1%) represents the metabolically active pool. Plasma Ca concentrations are particularly maintained by hormones controlling bones metabolism and do not show uniform variations after acute exercise. In regard to chronic energy restriction special attention has to be drawn to the problem of "athletic osteoporosis" [46, 177]. Under-weight athletes - particularly females - often show calcium intakes lower than recommended. In addition, urinary calcium loss rises in athletes consuming a high protein diet, which is generally accompanied by high phosphorus intake from protein sources. However, the role of an inadequate calcium intake in the pathogenesis of stress fractures and reduced bone density in athletes is not clear; on the contrary there is agreement that the aetiology of the "athletic osteoporosis" has been associated with depressed levels of plasma hormones, e.g. GnRH, sex hormones and leptin, which influence bone metabolism. No prospective studies about the benefit of calcium supplements in the prevention of stress fractures or bone density in athletes are available.

9.3. Trace elements

As an important constituent of oxygen and electron binding molecules, iron is a significant element in aerobic metabolism and energy production. As in normal populations a poor iron status can be diagnosed in athletes by routine clinical and biochemical markers without problems. However, the term "sports anaemia" is very commonly, but often not appropriate used in sports and physical performance medicine. In a representative sample the prevalence of anaemia, documented by plasma ferritin concentrations, is nearly the same in athletes as in controls [10]. Due to the harmful effects of iron overload to the organism [155] iron supplements are no longer recommended for self-medication in athletes. Iron preparations should only be prescribed to athletes with clinically diagnosed anaemia.

There are indications that Zn-intake in athletes is marginal. About 20% of the examined endurance athletes [6, 12] did not reach the 10-mg/d-zinc intake level according to their dietary records. Further research is indicated because clinical experience showed that objective symptoms of zinc deficiency occurred when the daily supply chronically fell below 10-mg/d [123]. In addition, studies have shown a positive correlation between urine Zn losses and systematic stress parameters, such as cortisol and interleukin 6 (IL-6) [12, 88]. These observations are of importance since Zn is essential for immunological functions [33, 88]. Although no clear Zn deficiency has been reported in athletes, it is suggested that the increased risk for URTI in athletes could be related to the Zn status [116]. Further studies are warranted.

The trace elements copper (Cu), selenium (Se), and manganese (Mn) are significantly involved in physical performance playing a role in energy and free radical metabolism [37, 88, 139]. Unfortunately, because of analytical difficulties and missing clinical routine, data about balances of

these trace elements in athletes are not representative, and in addition, specific recommendations for trace element intake in athletes other than the usual recommended dietary allowance for healthy people [41, 48] have not been published. Trace elements, particularly Cu, are lost in significant amounts in sweat - that means at a range up to the dietary requirement. Sustained exercise-induced sweating may lead to an increased loss of trace elements in general [11].

As an essential component of the enzyme glutathione peroxidase, Se is involved in the regulation and breakdown of hydroperoxides [151]. Thus, Se may play a significant role in the prevention of free radical damage and oxidative stress, even in situations of muscular stress and exhaustive exercise [154]. Se acts similarly to vitamin E, and vitamin E deficiency synergistically augments symptoms of Se deficiency. Muscular discomfort or weakness is documented after continuous periods of selenium-free diets (e.g. parenteral nutrition) [151]. In conditions of Se deficiency significant limitations of cellular anti-oxidative properties can be observed. However, controlled studies concerning the impact of Se supplementation on lipid peroxidation in athletes are not yet available.

The trace element Mn is an essential component of the mitochondrial metallo-enzyme superoxide dismutase (SOD) and is involved in the regulation of free radical metabolism [93]. Animal studies have shown that the activity of this mitochondrial enzyme may be regulated by the dietary Mn intake. However, well-controlled experimental data in athletes are not available.

Data about the nutritional requirements and essentiality of trace elements such as cobalt, nickel, silicon, boron, lithium, tin and vanadium in athletes are weak or not available.

9.4. Vitamins

In the past decades several studies have addressed the issue whether athletes have problems related to dietary vitamin intake and tissue levels. Reviews on this topic uniformly conclude that data on plasma vitamin levels and enzymatic stimulation tests in athletes show the same incidence of marginal or deficient levels as observed in sedentary populations. It is generally accepted in the scientific community that with an adequate dietary intake, there is no further need for supplementation, since supplementation studies with vitamins do not show an improvement in performance [156].

9.4.1. Vitamins involved in energy metabolism (Vitamin B1, Vitamin B2, Vitamin B6, Vitamin B12, Niacin)

As far as their biochemical functions are concerned, these vitamins play an essential role in energy metabolism and consequently physical performance. Results from studies concerning the vitamin status of athletes as well as studies examining for the ergogenic effects of extra vitamin

supplementation have not yielded any support to recommend intakes beyond an adequate balanced diet [1, 69, 84, 148, 174, 174 A, 178].

Recommended daily intake is set at 0.5 mg vitamin B1/1000 kcal, at 0.6 mg vitamin B2/1000 kcal, at 6.7 mg niacin/1000 kcal energy, and at 0.02 mg vitamin B6/g protein intake [41] in normal subjects.

9.4.2. Antioxidants (Vitamin C, Vitamin E and carotenoids)

Exercise results in an increased production of free radicals. There is growing evidence that exercise-induced free radical production could result in muscle fatigue and contribute to the late phase of exercise-induced muscle injury [128]. To defend against radical damage, two important classes of endogenous protective mechanisms work together in the muscle cell: 1) the endogenous enzymatic antioxidants such as Super Oxide Dismutase (SOD) and catalase and the non-enzymatic exogenous antioxidants including vitamin C, E, Beta-Carotene and ubiquinones. A high level of training results in a higher level of endogenous anti-oxidative capacity [138]. In this regard, high intensity exercise training is superior to low intensity exercise in upregulation of the endogenous enzymatic systems [128]. So far, limited information is available on the effects of endurance training on the exogenous antioxidants. There are some indications that regular exercise improves the antioxidant reserve such as vitamin E in skeletal muscle. However the results are not consistent [77].

Numerous studies with both antioxidant supplemented and antioxidant deficient diets have been carried out to understand the role of dietary antioxidants in oxidative stress [138]. In this context mainly vitamin E was tested. In contrast to studies of the 70's more recently published data did not find significant effects of the antioxidative vitamins C, E and beta carotene on physical performance in well designed controlled and double-blind studies [38, 64]. Moreover, there is no agreement on the hypothesis that antioxidants, predominantly vitamin E, can prevent an increase of lipid peroxidation and prevent oxidative muscular damage following physical exercise [120]. Since vitamin E can reduce lipid peroxidation, it is assumed that this vitamin may protect against oxidative damage and exercise-induced inflammation. Recently published data in a homogenous group of athletes [14] indicate that neither the exercise-induced peroxidation rate, expressed by diene production, nor the muscular cell damage, expressed by an increase in serum creatine kinase activity, is related to the individual plasma vitamin E concentration.

9.5. Essential fatty acids

Essential fatty acids are components of cellular membranes and structures; they significantly influence the plasticity and rigidity of muscle and blood cells, both of which are stressed by exhaustive aerobic exercise [13, 86] Data about membrane fluidity and oxygen diffusion in relation to membranes PUFA distribution in athletes have been published [51, 80]. So far, results about improved aerobic capacity with PUFA supplementation are not available. The longchain PUFA's, particularly eicosapentaenoic acid (EPA, fish oil) and gamma-linolenic acid (γ-LA, evening

primrose oil), can weaken the inflammatory response to physical stress by modulating the eicosanoid pathway, studies have shown that both muscular and systemic inflammatory stress can be influenced in apparently healthy individuals including endurance athletes, by modulating the composition and quality of dietary fat [87]. Within the trend for a lower fat intake of about 30% of total energy intake, athletes should pay greater attention to the intake of PUFAs and essential fatty acids. It is recommended that the omega-6 to omega-3 ratio be maintained at about 5 to 1.

9.6. Composition and specifications for supplements in particularly minerals, trace elements, vitamins and essential fatty acids

The committee is of the opinion that specific mineral and/or vitamin food supplements do not satisfy any additional particular physiological needs of individuals involved in intense muscular exercise taking an adequate dietary intake into consideration.

For essential fatty acids the scientific literature do not allow any recommendation at this moment.

10. Category D.II. Other food components

10.1. Caffeine

Caffeine is known as one of the most widely used non-nutritive components in beverages in the Western world and is therefore already present in the diet of many athletes. Already for a long time caffeine is considered as a nutritional ergogenic aid in physical performance. However, only in the last decade have a number of well-controlled studies clearly demonstrated its efficacy in relation to prolonged endurance exercise as well as short term intense exercise [143].

At present, the mechanism by which caffeine acts is not well known. Three major theories for the ergogenic effect have been suggested.

The first theory involves a direct effect on the sympathetic nervous system (SNS), leading to a stimulatory effect on the neural signals between brain and neuromuscular junction.

The second theory proposes a direct effect on the skeletal muscle metabolism by increasing, among others, cyclic AMP.

The third and most accepted theory involves an increase in fat oxidation, sparing endogenous carbohydrate stores and thus improving performance especially in exercise where CHO availability limits performance. The evidence demonstrating that caffeine is an ergogenic substance has forced the IOC to set a limit to prevent extreme use of this component. Caffeine is a restricted substance for athletes in competition that allows up to 12-ug caffeine/ml urine. This level would only be approached by an excessive intake of more than 6 regular cups of drip-percolated coffee. There is no restriction in training. In a well-controlled study, intake of 9 and 13 mg/kg body weight caffeine resulted in urine levels above the doping limit in some individuals [122].

However, caffeine ingestion even at low levels i.e. 3-8 mg/kg body weight prior to exercise, enhances performance of both prolonged endurance exercise and short-term intense exercise lasting approximately 5 minutes in the laboratory [143]. Recently it was also demonstrated that carbohydrate-electrolyte solutions with low concentrations of caffeine (2.1, 3.2 and 4.5 mg/kg BW respectively) improve endurance performance with low post-exercise urinary caffeine concentrations (1.3; 1.9 and 2.5 ug/ml respectively) [90].

10.2. Creatine

Creatine is a non-essential dietary compound found in high abundance in meat and fish. It is synthesized within the body, primarily in the liver, from the amino acids arginine and glycine. Diet and endogenous synthesis each contribute about half in subjects on a normal diet [172].

Creatine phosphate (CrP) serves as a readily available source of energy in skeletal muscle and other tissues [110, 109]. The rapid re-phosphorylation of ADP from CrP via the Creatine kinase reaction buffers changes in ATP during transitions between rest and exercise, and contributes a substantial fraction of ATP synthesis during short duration, high intensity exercise.

The relative importance of CrP during exercise is dependent on the nature of the exercise. For most exercise situations, the demand for ATP is predominantly provided through oxidative phosphorylation in the mitochondria. However, when aerobic energy production cannot meet the demand for ATP, anaerobic energy production from CrP hydrolysis and glycogenolysis/glycolysis is required to assist in the provision of ATP [143]. Such cases include the transition from rest to exercise, the transition from one power output to a higher power output, and power outputs above 90-100% maximal oxygen consumption (VO_2max). During a bout of high intensity exercise the relative importance of CrP hydrolysis to ATP synthesis falls off dramatically as the exercise duration is increased beyond a few seconds.

Research [31, 54, 70, 72, 133] has shown that creatine ingestion increases the total creatine content in human muscle by approximately 15-20% (mean value). Such increases can be achieved by ingestion of 20 gram per day for 4-5 days, but also by ingestion of 3 gram per day over a period of 1 month [72]. The increased creatine content is maintained when the ingestion is reduced to 2 gram per day after the original loading period [72]. The increase in creatine content is rather variable between subjects, ranging from zero to up to 40% [68]. Thus, there are 'responders' and 'non-responders.'

Sub-maximal exercise performed prior to creatine ingestion can augment muscle creatine accumulation by approximately 10%, but again the variation in response is marked among individuals [72]. Muscle creatine accumulation can be substantially augmented by ingesting creatine in combination with large quantities of simple carbohydrates [66, 67].

Coincident with the retention of creatine, there is a substantial reduction in urine production on the first 3 days of the loading period [72]. This retention of water is thought to be related to an osmotic load caused by creatine retention and to account for the rapid-onset weight gains experienced by many individuals ingesting creatine. Many studies have reported increases in body mass of 1-3 kg following short-term (5-7 days) creatine supplementation [149].

Short-term creatine supplementation (5-7 days of ~20 g/d) can lead to an improvement in performance. Most but not all of the studies indicate that creatine supplementation significantly enhances the ability to produce higher muscular force and/or power output during short bouts of maximal exercise in healthy young adults [149]. At present, exercise performances that are improved include: various protocols of short-term, all-out cycling, sprinting, repeated jumping, swimming, kayaking/rowing, and resistance exercise performance. Interestingly, the greatest improvements in performance seem to be found during a series of repetitive high power output exercise bouts [149]. Exercise performance during the latter bouts of a series (e.g., third, fourth, fifth) can be increased by 5-20% over that measured for the placebo group. These experimental protocols employed exceptionally high power output efforts (e.g., maximal cycling and/or power jumping that can be maintained for only a short period, usually seconds) separated by fairly brief periods of rest (e.g., 20-60 seconds). These are the exercise conditions where the transitional energy contribution from CrP is likely most significant; further, the

short-term rest periods between bouts are apparently sufficient to permit an enhanced recovery of the muscle CrP concentration in those individuals with a greater total creatine concentration as was shown in an in vivo magnetic resonance spectroscopy study [89]. It, therefore, is likely that creatine supplementation improves exercise performance in sport events that require explosive, high-energy output activities especially of a repeated nature.

In some [100] but not all studies, creatine supplementation increased maximal isometric muscle strength and not alter the rate of maximal force production [157, 161]. Creatine supplementation also did not appear to enhance aerobic-oriented activities [3, 158].

Few data exist on the long-term effects of creatine supplementation. A number of studies indicate that creatine supplementation in conjunction with heavy resistance exercise training (e.g., 4 to 12 weeks in duration) enhances the normal physiological adaptations to a weight training program [149]. Typical training adaptations including, increases in body mass fat-free mass, maximal strength and power, lifting volume, and muscle fiber hypertrophy, are all significantly enhanced concurrent with creatine supplementation.

The loading doses suggested by the manufacturers are 10-20 g/day for 5-7 days and then 2-5 g/day as a maintenance dose.

There are numerous anecdotal reports of creatine supplementation causing gastrointestinal, cardiovascular and muscular problems. There is no scientific evidence to support these reports. However, at this moment in time it also cannot be concluded (documentation is lacking or incomplete) that creatine supplementation is free from health risks [149]. Creatine ingestion prior to competition in the heat should be discouraged as it may interfere with water absorption and as there is no rationale for intake immediately before competition (despite such claims on some commercial preparations). Creatine supplementation increases urinary creatine and creatinine excretion [70, 72]. Thus, it would be expected that creatine supplementation will increase plasma creatinine concentrations in healthy individuals; there is no a priori reason to expect that acute and long-term creatine ingestion impairs kidney function. This has been confirmed recently in experimental studies [126, 127].

10.3. Carnitine

The main sources of L-carnitine are red meats and dairy products in the diet and endogenous biosynthesis from trimethyllysine and methionine in liver and kidney [21]. Healthy humans produce enough carnitine to maintain normal bodily functions, even when the diet contains no carnitine. About 98% of the carnitine of the human body is present in skeletal muscle and heart. In the human body carnitine functions to transport long-chain fatty acids across the mitochondrial inner membrane so that the fatty acids can be subjected to Beta-oxidation and used for ATP production in the mitochondrial matrix. Another function of the carnitine pool in the muscle is to store excess acetyl groups and keep free CoA available for muscle metabolism during high intensity exercise, when the pyruvate dehydrogenase complex is maximally

activated [42]. Carnitine containing supplements are widely available in health food and sport nutrition shops and are used by athletes in an attempt to improve performance and by obese subjects to try to burn fat and lose weight. However, oral carnitine supplementation in humans for periods of 2-3 weeks in amounts of 5-6 gram per day did not increase the carnitine concentration in muscle [5, 152, 166] and carnitine supplementation, therefore, can not have an effect on muscle metabolism at rest or during exercise. In agreement with this conclusion, controlled studies [167, 171, 71] do not support the commercial claims that carnitine supplementation: 1. Helps to lose weight or reduce body fat mass; 2. Increases fat oxidation and reduces glycogen breakdown during prolonged cycling or running; 3. Increases VO_{2max} and reduces lactate accumulation during maximal and supramaximal exercise; and 4. Improves endurance performance. In conclusion, the scientific data available do not support the use of carnitine supplements in relation to physical performance.

10.4. Medium-Chain Triglycerides (MCT)

In theory addition of a fat source to a carbohydrate containing solution could increase plasma FFA availability and oxidation in muscle and thus spare muscle glycogen breakdown and improve performance. This effect, for various reasons (slow gastric emptying, slow absorption, inhibition of long-chain fatty acid oxidation by glucose ingestion), cannot be reached by co-ingestion of carbohydrate (CHO) and long-chain triglycerides (LCT) immediately before or during exercise. Several studies, therefore, investigated whether co-ingestion of CHO and medium-chain triglycerides (MCT) could have a performance effect via this mechanism. MCT ingested orally immediately before and during exercise, in contrast to LCT, did not reduce gastric emptying [7] and, therefore, did not reduce the availability of the co-ingested glucose. MCT are rapidly absorbed by the intestine, directly into the portal vein as medium-chain FFA [171] and are rapidly oxidized after absorption [47, 137, 108, 78]. Probably part of the MCT oxidation occurs in the liver and part in the exercising muscles after conversion to ketone bodies. Only one study [163] observed muscle glycogen sparing and a positive effect on time trial performance when 86 g of MCT was added to a CHO drink and ingested in small repeated boil during 2 h of endurance exercise. Several other studies [78, 79] failed to find endogenous glycogen sparing and a performance effect when 30 to 86 g of MCT was added to CHO containing solutions ingested during exercise. In several of these studies MCT ingestion alone and in combination with CHO caused severe gastrointestinal problems and interfered with the outcome of the performance tests. In all studies ingestion of MCT alone had no or a negative effect on performance, often as a consequence of gastrointestinal cramping.

10.5. Branched Chain Amino Acids (BCAA)

Although the subjective sensations of fatigue that accompany prolonged exercise are generally considered to be the result of events occurring in the muscles or the cardiovascular system, there is

growing evidence that the signals that arise in the periphery are modulated by events occurring within the central nervous system [45]. Newsholme and colleagues [20] proposed that an increase in brain serotoninergic activity was a cause of central fatigue during endurance exercise. Increases in brain 5-hydroxytryptamine (5HT) could result from an increase in the transport of the precursor tryptophan (Trp) from the plasma across the blood-brain barrier. Increasing the plasma concentration of the branched chain amino acids (BCAA), which are competitive inhibitors of Trp uptake, could reduce brain 5HT accumulation, and these observations have led to suggestions that BCAA should be added to drinks intended for consumption during prolonged exercise.

Controlled studies to improve performance by the administration of BCAA during exercise at ambient temperature all failed to demonstrate the claimed effect [172]. Mittleman et al., [111] observed an ergogenic effect during exercise in the heat, but this isolated finding in a special condition does not prove that BCAA supplementation could improve endurance performance in other situations.

10.6. Composition and specifications for supplements in particular caffeine, creatine, carnitine, MCT and BCAA

Controlled scientific studies investigating claimed mechanisms and performance do not provide a basis for the use of carnitine, MCT and BCAA intended as nutritional ergogenic aids, to meet expenditures of intense muscular efforts and especially for sportsmen.

Caffeine ingestion at levels of 2-8 mg per kg body weight prior to and during exercise enhances performance during prolonged endurance exercise as well as during short-term (approximately 5 minutes) intense exercise.

Creatine ingestion leads to a small improvement in exercise performance in sport events that require explosive, high-energy output activities especially of a repeated nature. The general recommended advised ingestion protocol is 5 days of creatine loading (10-20 gram per day in 2-4 equal portions) followed by a maintenance dose of 2-3 gram per day. Ingestion of larger amounts for more prolonged periods may not be safe [149].

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The Effects of Creatine Dietary Supplementation on Anterior Compartment Pressure in the Lower Leg During Rest and Following Exercise

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Objective: To examine the effects of creatine supplementation on anterior compartment pressure of the lower leg at rest and following exercise.

Design: 14 college-age males received creatine or placebo supplementation for 34 days. At baseline, anterior compartment pressure was measured preexercise, immediately postexercise, and 1, 5, and 15 minutes postexercise after a level treadmill run for 20 minutes at 80% of maximal aerobic power.

Intervention: Following baseline testing, subjects began a 6-day creatine or placebo loading phase at a dosage of 0.3 g · kg body mass^{-1} · d^{-1}. This was followed by a 28-day maintenance phase at a dosage of 0.03 g · kg body mass^{-1} · d^{-1}. Subjects and investigators were blinded as to treatment administration. Subjects continued to exercise during the supplementation period. After 6 days and 34 days of supplementation, anterior compartment pressure was measured at rest and following exercise.

Results: Creatine supplementation for 6 days significantly increased compartment pressure compared with the placebo group at rest (76%), immediately post- (150%), 1 minute post- (125%), 5 minute post- (106%), and 15 minute postexercise (77%). Anterior compartment pressures continued to remain significantly higher for the creatine group compared with the placebo group at rest (72%), immediately post- (125%) and 1 minute postexercise (180%) after 34 days of creatine supplementation.

Conclusions: These data indicate that creatine supplementation abnormally increases anterior compartment pressure in the lower leg at rest and following 20 minutes of level running at 80% of maximal aerobic power.

Key Words: Creatine—Compartment pressure syndrome—Exercise—Ergogenic aids—Side effects.

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INTRODUCTION

Most lower leg problems in active individuals can be attributed to repetitive and overuse injuries. Three main etiologies that typically arise are the medial tibial stress syndrome (shin splints), stress fractures, and exercise-induced compartment compression syndromes. The deep posterior and anterior regions of the lower leg comprise the majority of cases diagnosed as exercise-induced compartment compression syndrome,[1–10] and increasing compartmental pressures may result in decreased exercise capacity and ultimately may require medical intervention.

Creatine supplementation is believed to improve exercise performance and recovery,[11–13] and thus may serve as an ergogenic aid in some types of athletic performance,[11,14–19] but not all.[16,20–22] Intramuscular stores of creatine and phosphocreatine can be increased by using creatine as a dietary supplement.[12,13,23] However, the side effects associated with creatine supplementation have not been fully defined. For example, creatine supplementation has been associated with negative side effects such as gastrointestinal distress and muscle cramping.[24,25]

Creatine loading causes increased body mass of 0.9–3.8 kg[11,14–19] that is most likely the result of increased total body water[14–16] and/or increased protein synthesis.[26,27] Indeed, decreased water excretion with concomitant water retention has been documented in the early phase of creatine supplementation.[28,29] The water retention has been speculated to be involved with the cotransport of creatine and sodium into muscle fibers.[30,31] Whereas the mechanism for creatine-induced protein synthesis has not been clearly established, previous work by Ingwall has shown that cardiac and skeletal muscle protein synthesis can be increased by creatine.[26,27]

In our clinical practice and in our work with sports

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teams we have observed increases in muscle size and lower leg pressures in individuals consuming creatine. We have recently reported acute anterior compartment syndrome in one young male consuming creatine monohydrate as a supplement. It was of interest to further define the potential implications of creatine supplementation on anterior compartment pressure during rest and following exercise. We report here the effects of 34 days of creatine dietary supplementation on lower leg anterior compartment pressure at rest and following 20 minutes of treadmill running.

METHODS

Subjects

Fourteen healthy, physically active male subjects (ages 19–25 years) participated in a randomized, double-blind examination of anterior compartment pressures of the lower leg following creatine or placebo dietary supplementation. Subjects were excluded from the study if they were vegetarian dieters, used prescription medications and/or anabolic steroids, had a resting blood pressure $\geq$ 139/89 mmHg, or had used creatine supplementation within 3 months of starting the investigation. Subjects were also screened for exclusionary criteria consisting of a previous history of lower leg etiologies involving the musculoskeletal, neurological, and/or vascular structures. All subjects read and signed an informed consent form and completed a health history questionnaire in accordance with guidelines set forth by the Advisory Committee for Human Experimentation at the University of Kansas.

Experimental Design

The subjects were tested on four occasions. Initial testing determined body composition and maximal aerobic power and familiarized the subject with the treadmill testing protocol. For the experimental exercise sessions, subjects reported to the laboratory in a 3-hour postabsorptive state having refrained from exercise for 48 hours, and from caffeine, alcohol, and other potential diuretics for 24 hours prior to testing. During each experimental testing session, subjects ran on a level treadmill at 80% of maximal aerobic power for 20 minutes. Anterior compartment pressure was measured at rest and following the run. After baseline compartment pressure was recorded, the subjects were randomly assigned to a creatine or placebo group and began the creatine dietary supplementation.

Creatine Supplementation

Subjects received either creatine (Nutrasense, Shawnee Mission, KS, U.S.A.) in tablet form (1 g creatine monohydrate and 1.4 g dextrose) or a placebo (1 g dextrose) matched for size and taste. Subjects were given 6 days of creatine or placebo in a loading phase (0.3 g · kg body mass^{-1} · d^{-1}) followed by 28 days of creatine or placebo in a maintenance phase (0.03 g · kg body mass^{-1} · d^{-1}).[23] Subjects consumed the supplement in four equal dosages throughout the day according to the manufacturer's directions. Subjects continued training during supplementation. Prior to and after completion of 6 days and 34 days of supplementation, muscle biopsies from the *vastus lateralis* were collected and anterior compartment pressure of the lower leg was measured at rest and following exercise as described below.

Preexercise Testing Measurements

Prior to each exercise testing session, body mass, body fat percentage, resting blood pressure, and lower leg volume were recorded. Body mass was determined using an electronic scale and body composition was measured via a seven-site skinfold. After the subject was seated for 15 minutes, resting blood pressure was measured by the same research technician using a mercurial sphygmomanometer. Systolic and diastolic pressures were measured two times and averaged. Lower leg volume was determined via six anthropometric measurements and two skinfolds taken from the right leg. A Gulick II tape was used to measure leg circumferences at 90° to the longitudinal axis of the lower leg. The sites measured were the minimal ankle circumference, midcalf circumference, middistance between the ankle and calf markers, subpatellar circumference, middistance between the calf and subpatellar circumference, and the joint line circumference. Skin-fold thickness was measured with Lange calipers at two sites at the maximum circumference level of the medial and lateral calf. Two standard diameters (tibia and femoral intercondylar) were measured by standard anthropometric calipers. Three measures were taken at each site and the mean values used in the calculation of leg volume.[32]

Compartment Pressure Measurements

Measurements of resting and postexercise compartment pressures were obtained at baseline and after 6 days and 34 days. Anterior compartment pressure was measured in supine subjects using a slit catheter and a Stryker Intra-compartment Pressure Monitor System (Kalamazoo, MI, U.S.A.). A towel supported the knee with the ankle in a neutral position, the first toe of the foot pointed vertically.[33] Resting pressures were taken when the musculature of the anterior compartment felt relaxed upon palpation.[33] The point of catheter insertion into the anterior compartment was 15 cm distal to the tibial tuberosity and 2 cm lateral to the tibial crest.[8] A 1 cm^2 area of skin was sterilized with betadine solution and anesthetized with 1 ml of 1% lidocaine without epinephrine. The fascia was pierced at a 30° angle to the long axis of the leg and the catheter was directed cephalad at a 45° angle into the muscle belly of the tibialis anterior and positioned 1–4 cm within the anterior compartment. The pressure monitor was filled with a heparinized saline solution that provided a continuous fluid column from the catheter tip to the transducer. Following catheter insertion, the pressure was allowed to stabilize for 30 s and then recorded from the digital display monitor. Following measurement of resting pressure, the catheter was removed and the subject began exercising. After a 5-minute warm-up at 50% of maximal aerobic power, the

exercise intensity was increased to 80% of maximal aerobic power, and the subject ran for 20 minutes. This exercise protocol is typically used in our clinical practice to assist in the diagnosis of anterior compartment syndrome. Immediately after exercise, each subject was positioned as previously described and the catheter was reinserted. Pressures were recorded immediately postexercise, and 1, 5, and 15 min postexercise.

Tissue Collection and Analysis

Muscle biopsies from the *vastus lateralis* were performed at baseline and after 6 days and 34 days of supplementation using procedures outlined by Bergström[34] with suction applied to the biopsy needle to aid in tissue extraction.[35] The samples were immediately frozen in liquid nitrogen and stored at −70° until analysis. The freeze-dried sample was powdered and analyzed for adenosine triphosphate (ATP), phosphocreatine, and creatine.[28] Total creatine was calculated as the sum of phosphocreatine and free creatine.

Statistical Analysis

Mean and standard deviation were calculated for all data. A three factor (group by period by time) repeated measures analysis of variance (ANOVA) was used to identify significant differences in anterior compartment pressure. Post hoc tests were performed if significant F values were obtained. For secondary outcome measures such as body mass and muscle creatine levels, we used a two factor (group by time) repeated measures ANOVA, with appropriate post hoc tests, to identify significant differences after 6 days and 34 days of supplementation.

RESULTS

Preexercise Measurements

No statistically significant differences were observed between the creatine and placebo groups at baseline, 6 days, or 34 days of supplementation for body mass, body fat percent, resting blood pressure, or leg volume (Table 1). Significant gains in body mass, ranging from 1.0 to 2.2 kg, were observed at 6 days in the creatine group. Body mass in the creatine group remained slightly elevated (1.1 kg) at 34 days. In addition, blood pressures were slightly higher after creatine supplementation, as were leg volumes, but these values were not significantly different from baseline.

Anterior Compartment Pressures Changed with Dietary Supplementation

Compared with placebo, the creatine-supplemented group showed higher compartment pressures at rest, and markedly steeper increases in response to exercise. Individual data are presented in Figures 1, 2, and 3, and group means summarized in Table 2. Before initiation of dietary supplementation, preexercise values between the two groups were not significantly different and were within normal ranges for resting and postexercise values. In contrast, significant increases in mean pressures were observed in the creatine group following both 6 days and 34 days of dietary supplementation (Table 2). Increases were observed at rest, immediately postexercise, and at 1, 5, and 15 minutes postexercise at both 6 and 34 days of supplementation. In some instances the increase in pressure was rather marked. For example, compared with placebo, the resting compartment pressure was increased by an average of 76% after 6 days, and by 72% after 34 days of supplementation. After 6 days of supplementation, subjects in the creatine group had immediately postexercise pressures that were 150% higher than those of the placebo group. After 34 days, creatine subjects had pressures averaging 125% above placebo for the immediate postexercise measurement.

For two of the subjects, the increase in compartment pressure was greater at 34 days than at 6 days, but in most cases, the magnitude of the increase was similar at 6 days and 34 days, suggesting that the maximum effect

TABLE 1. *Effect of creatine and placebo supplementation on body mass, body fat percent, resting blood pressure, and leg volume at baseline and after 6 and 34 days of creatine dietary supplementation*

	Baseline	6 days supplementation	34 days supplementation
Body mass (kg)			
Creatine	78.7 ± 12.6	79.7 ± 12.4	79.5 ± 12.9
Placebo	77.2 ± 9.4	77.4 ± 9.5	77.6 ± 9.6
Body Fat Percent			
Creatine	11.6 ± 4.2	11.8 ± 4.2	11.8 ± 4.2
Placebo	10.8 ± 5.1	10.8 ± 5.1	10.8 ± 5.1
Systolic BP (mmHg)			
Creatine	121.1 ± 8.9	128.4 ± 11.9	128.0 ± 8.2
Placebo	121.1 ± 8.6	122.3 ± 13.3	122.0 ± 10.1
Diastolic BP (mmHg)			
Creatine	75.4 ± 7.5	76.6 ± 9.0	79.1 ± 4.7
Placebo	75.7 ± 8.4	75.7 ± 10.3	76.9 ± 8.4
Leg volume (ml)			
Creatine	1,118.9 ± 101.1	1,134.6 ± 102.0	1,128.0 ± 102.4
Placebo	1,097.7 ± 151.9	1,100.2 ± 156.8	1,100.0 ± 156.6

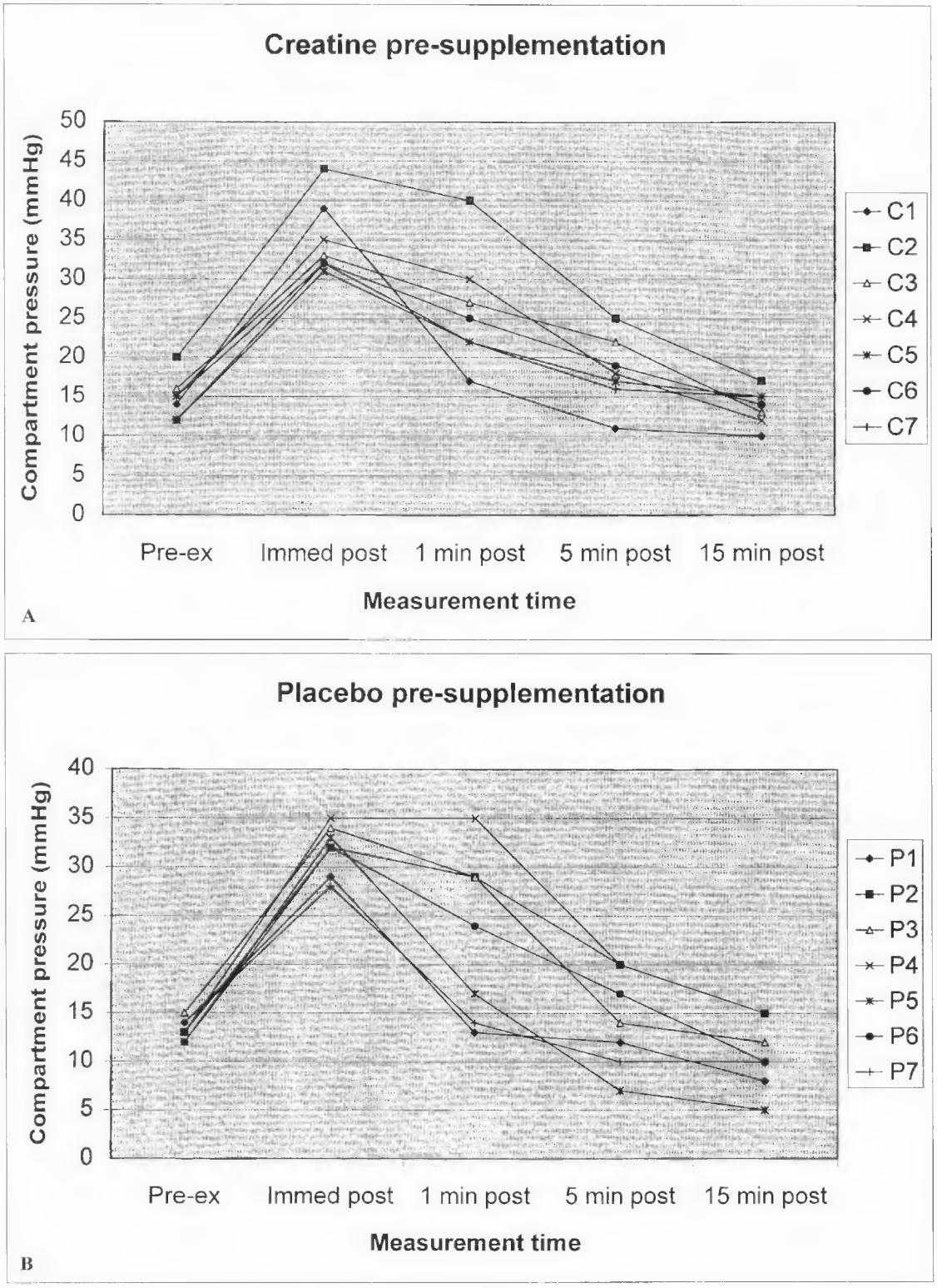
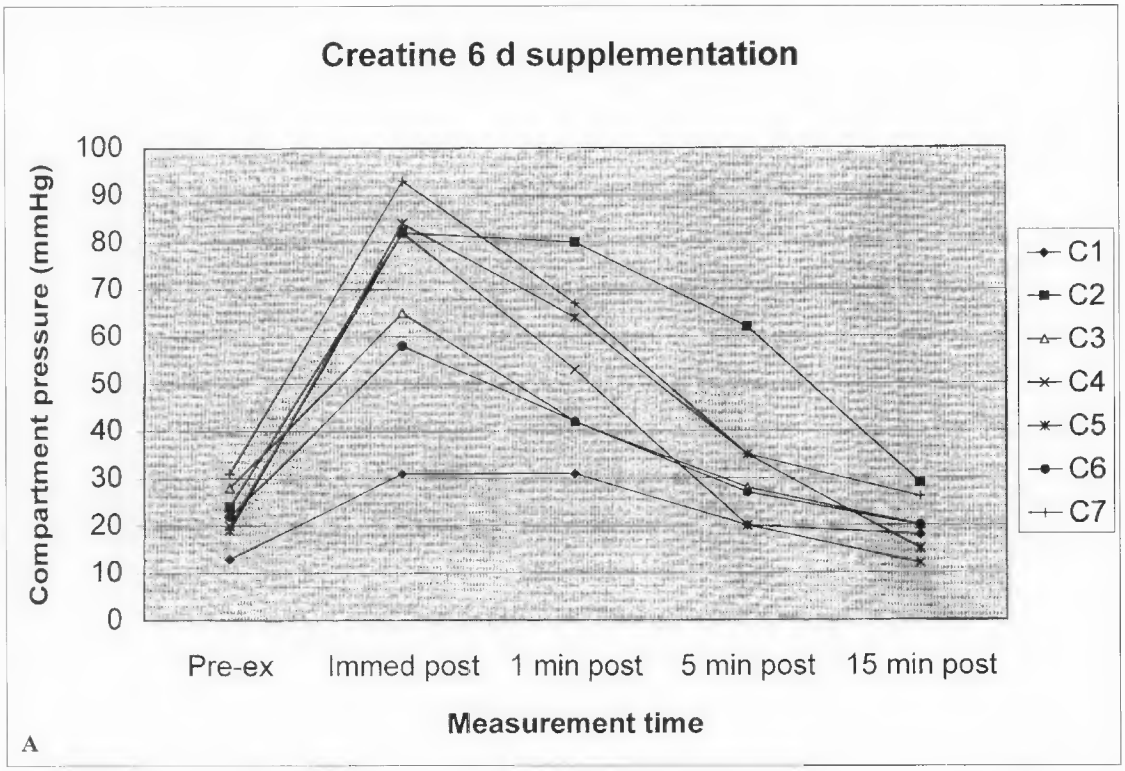


FIG. 1. Anterior compartment pressure prior to and following exercise in creatine (**A**) and placebo (**B**) supplement subjects at presupplementation.



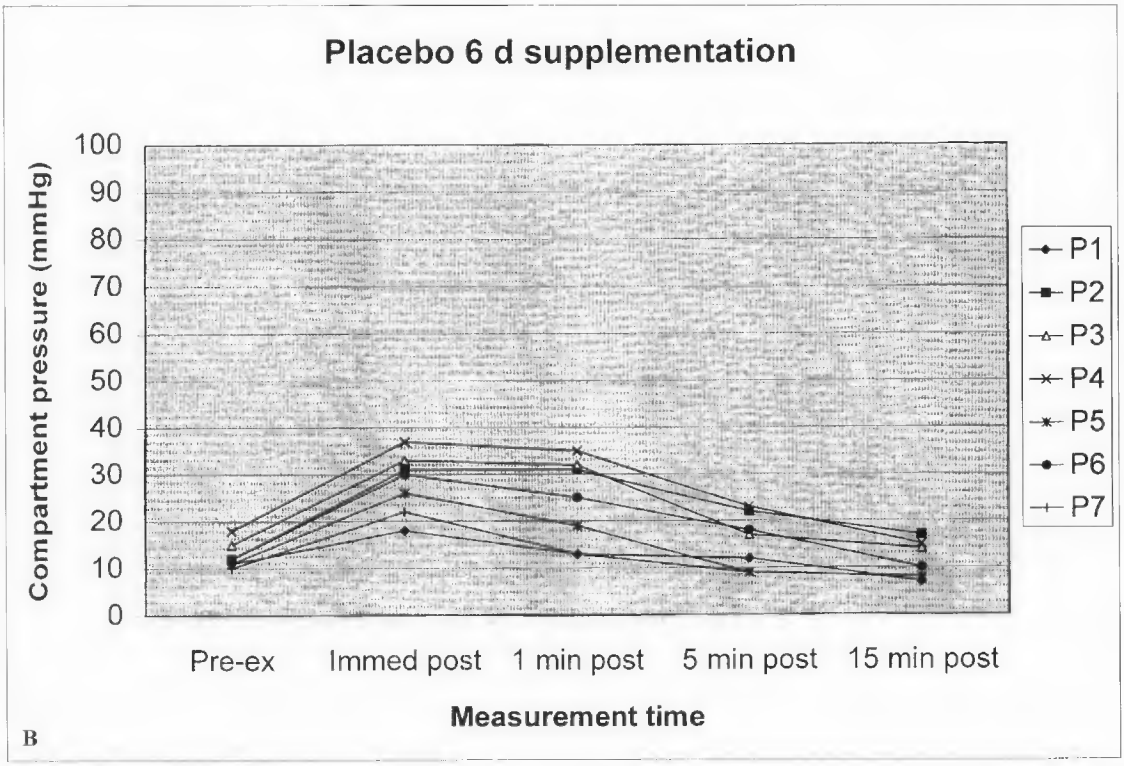


FIG. 2. Anterior compartment pressure prior to and following exercise in creatine (**A**) and placebo (**B**) supplement subjects after 6 days of supplementation.

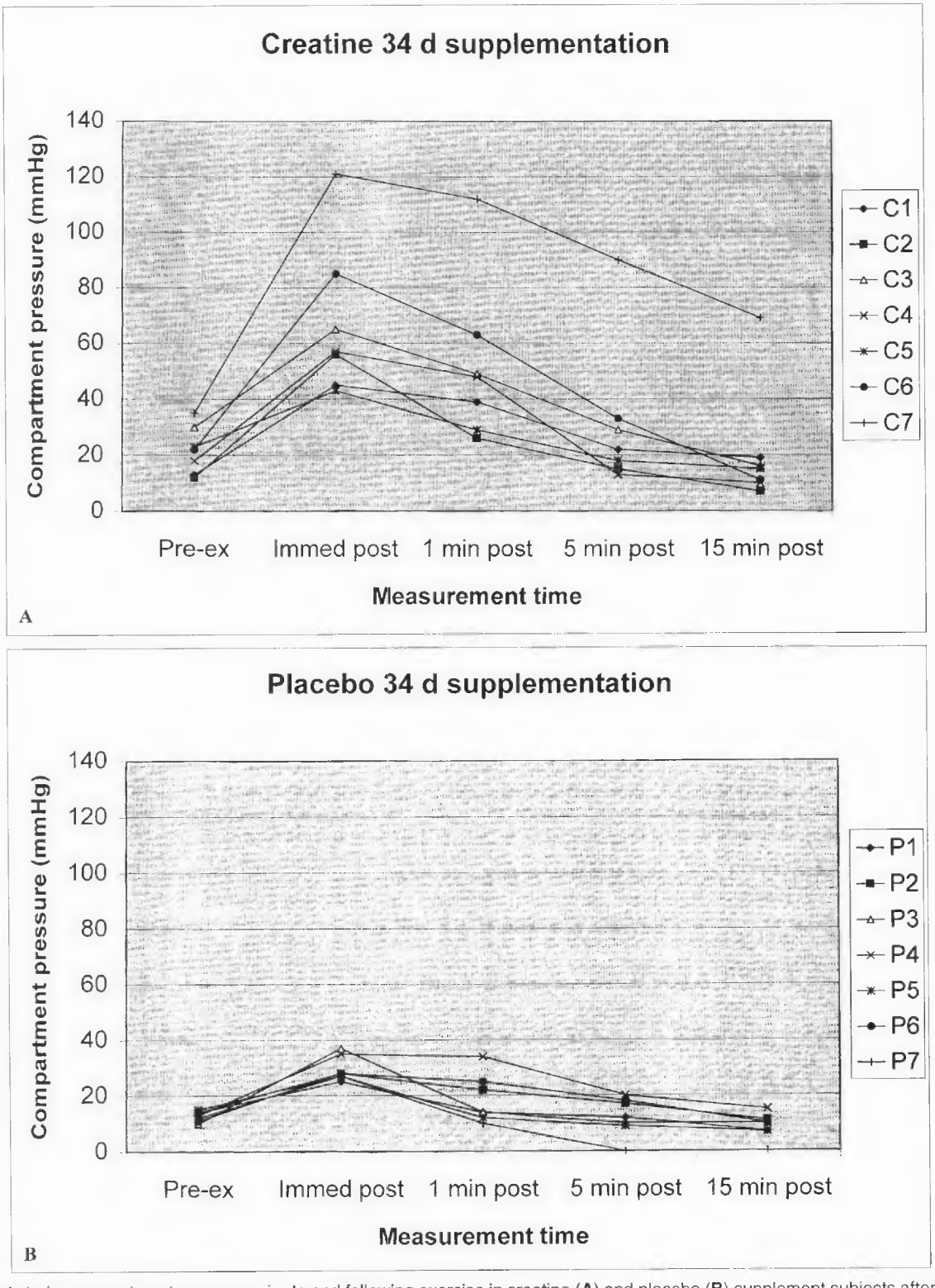


FIG. 3. Anterior compartment pressure prior to and following exercise in creatine (**A**) and placebo (**B**) supplement subjects after 34 days of supplementation.

was reached comparatively rapidly. In addition, a small subset of the subjects experienced a delay in recovery from the increased pressure as compared with controls. This is most apparent at 1 and 5 minutes after exercise (Figure 1), where the values show higher variability. By 15 minutes, most subjects' values had returned to preexercise levels, but differences between creatine and placebo groups were still observed.

TABLE 2. *Anterior compartment pressures (mmHg) measured at baseline, and after 6 days and 34 days of creatine dietary supplementation for preexercise, immediately post-, 1, 5, and 15 minutes postexercise*

	Preexercise	Immediately postexercise	1 min postexercise	5 min postexercise	15 min postexercise
Baseline					
Creatine	14.9 ± 2.7^{a}	35.1 ± 4.7^{b}	26.1 ± 7.4^{c}	18.3 ± 4.5^{d}	13.7 ± 2.3^{a}
Placebo	13.3 ± 1.1^{a}	31.9 ± 2.5^{b}	23.0 ± 8.5^{c}	14.3 ± 5.0^{a}	10.7 ± 3.6^{d}
6 d supplementation					
Creatine	22.4 ± 6.0	$70.7 \pm 21.2^{b,*,\#}$	$54.1 \pm 17.1^{c,*,\#}$	$32.4 \pm 14.4^{d,*}$	$20.0 \pm 5.9^{a,*}$
Placebo	12.7 ± 2.8^{a}	28.1 ± 6.6^{b}	24.0 ± 9.1^{c}	15.7 ± 5.8^{d}	11.3 ± 4.0^{a}
34 d supplementation					
Creatine	$21.9 \pm 8.5^{a,*}$	$67.4 \pm 27.5^{b,*,\#}$	$52.3 \pm 29.2^{c,*}$	31.4 ± 26.8^{d}	21.0 ± 21.5^{a}
Placebo	12.7 ± 1.8^{a}	30.0 ± 4.5^{b}	18.7 ± 8.7^{c}	12.3 ± 6.8^{a}	8.6 ± 4.6^{d}

[a,b,c,d] Within group differences ($p < 0.05$) across time for both groups at baseline, 6 days, and 28 days postsupplementation; same letter equals no difference.

* Between group (Cr and placebo) differences ($p < 0.05$) at each period and time of supplementation at baseline, 6 days, and 28 days postsupplementation.

[#] Within group (Cr) differences ($p < 005$) across periods of supplementation at baseline, 6 days, and 28 days postsupplementation.

Increased Muscle Tissue Creatine Content as a Result of Dietary Supplementation

As expected, the dietary supplement protocol used in this study was successful in increasing the intramuscular levels of creatine, phosphocreatine, and total creatine as compared with the placebo group (Table 3). Phosphocreatine increased by 7% and 10% at 6 and 34 days, respectively, compared with placebo, and creatine increased by 15% and 23%, while total creatine increased by 10% and 15%. No changes in muscle ATP were observed for either group.

DISCUSSION

Although creatine supplementation has been promoted as a successful ergogenic aid in the performance of athletic events, its effect on various health aspects has not been definitively determined. In order to completely characterize the effects of creatine supplementation, both positive and negative aspects must be elucidated.

It is believed that dietary creatine supplementation will improve athletic performance by increasing intramuscular creatine and phosphocreatine concentrations. In this study, as expected, the muscle tissue content for phosphocreatine, creatine, and total creatine was greater in the creatine supplementation group compared with placebo after 6 days and 34 days (Table 3). These muscle data are similar to that reported by Harris et al. who observed a 20% increase in phosphocreatine in subjects supplemented with 5 g of creatine 4–6 times per day for 1 week.[36] Similarly, Hultman et al. demonstrated that 20 g of Cr for 6 days would acutely elevate the total creatine content of muscle and that ingestion of 2 g Cr · day^{-1} for 30 days would maintain the elevated total creatine concentration.[23]

TABLE 3. *ATP, phosphocreatine, creatine, and total creatine concentrations (mmol · kg d.m.$^{-1}$) of the* vastus lateralis *at baseline, and after 6 days and 34 days of creatine dietary supplementation*

	Baseline	6 days supplementation	34 days supplementation
Adenosine triphosphate			
Creatine	24.1 ± 2.0	24.2 ± 2.1	24.3 ± 9.5
Placebo	24.6 ± 3.1	23.3 ± 1.3	24.7 ± 1.1
Phosphocreatine			
Creatine	78.0 ± 4.2^{a}	85.0 ± 3.6^{b}	84.4 ± 3.2^{b}
Placebo	81.2 ± 2.6	79.4 ± 4.1	77.0 ± 4.8
Creatine			
Creatine	37.3 ± 3.3^{a}	44.2 ± 4.3^{b}	45.9 ± 3.2^{b}
Placebo	40.7 ± 5.1	38.4 ± 4.7	37.2 ± 5.7
Total creatine			
Creatine	115.3 ± 6.2^{a}	129.2 ± 4.8^{b}	130.6 ± 7.8^{b}
Placebo	121.9 ± 6.0	117.8 ± 7.4	113.1 ± 8.3

[a] Significant differences ($p < 0.05$) within the creatine group; same letter equals no difference.

[b] Significant differences ($p < 0.05$) between groups.

A decrease in fluid excretion and an increase in fluid retention have been observed during the early phases of creatine supplementation.[23] In support of this, a sodium-dependent process that actively transports creatine across the cell membrane has been documented, which may be a factor in the observed changes in fluid homeostasis.[37,38] This mechanism allows for the uptake of one creatine molecule with two sodium molecules against a concentration gradient. Several studies report increases in body mass of between 0.9–3.8 kg during the early phases of creatine loading and suggest that the transport process is responsible for the increase.[15,17–19]

Creatine supplementation may contribute to the increased water transferred into the muscle fiber during the early phase of supplementation and as a result stimulate a swelling of the muscle fiber.[15] Additionally, the formation of new protein throughout the body following the later phase of supplementation may contribute to an increase in muscle fiber size.[39] Due to the rigidity of the anterior compartment of the lower leg, an increase in water content or de novo protein synthesis in the muscle fiber will likely result in higher anterior compartment pressures at rest and following exercise.

Three main factors should be considered when determining the presence of abnormally high tissue pressures specifically in the anterior compartment of the lower leg, including but not limited to: the geometrical dimensions

of the interstitial spaces, the physical characteristics of surrounding tissues, and the total amount of the fluid in the cell and in the interstitial spaces.[40] Previous research has documented an average increase of 20% in tissue volume of the lower leg compartments during exercise. This normal increase in volume results in part from an increase in regional blood volume and partly from increased transcapillary filtration of intravascular fluid.[41–43] However, if the compartment is unable to accommodate for this exercise-induced increase in fluid volume, pressure will rise within the osteofascial space. Any change in fluid homeostasis and/or increase in muscle fiber size as a result of creatine supplementation might further limit the space provided for normal increases in anterior compartment pressure. Due to this additional space restriction, fluid within the elastic compartment may well begin to shift from the vascular bed of the capillaries into the interstitial spaces. Capillary blood flow may be limited due to the increased pressures surrounding the vessel thus leading to an ischemic condition. This would be expected to be followed by a resultant decrease in microcirculation,[44] limiting the arterial and venous systems and further compromising nutrient blood flow, tissue oxygenation, neuromuscular function, and eventually leading to muscle necrosis.[45]

Recently, Robinson[46] reported a case of acute quadriceps compartment syndrome and rhabdomyolysis in a weight lifter using high-dose creatine supplementation. While a casual relationship could not be proven in the patient, it is interesting to note that the data reported in the current study lend support to the hypothesis that creatine supplementation may increase muscle compartment pressure.

Not only were the pressures that we observed higher at rest, as a group the creatine subjects demonstrated a significant increase in pressure following exercise. Even though the pressure responses did vary greatly among individuals, the patterns were similar over postexercise times measured following 6 and 28 days of supplementation. Following 6 days of supplementation, the resting and postexercise pressures were abnormally elevated and remained so at 34 days. In many subjects the higher pressures were observed in combination with a longer duration required for the pressures to return to preexercise levels. Clinically, the duration as well as the amount of the pressure is critical if these abnormal pressures remain high since this results in irreversible damage, particularly to the peroneal nerve.

Individuals suffering from increased pressures would be expected to complain of lower extremity aching, cramping, or burning pain and sometimes tightness over the affected compartment. Symptoms resembling these same complaints were noted in three individuals in the creatine group who were observed to have extremely high pressures at 6 and 34 days of supplementation. Subjective complaints of tightness and pain in the region of the anterior compartment during exercise were noted in these individuals and continued for approximately 10–15 minutes following exercise. Data from recent surveys also indicate that individuals consuming creatine as a supplement may experience additional adverse symptoms[24,25] such as diarrhea, muscle cramps, dehydration,[24,25] and gastrointestinal distress.[25] These findings suggest that despite the positive performance benefits that may result from creatine supplementation, the risk of side effects may lead to contraindications to exercise in some individuals.

CONCLUSIONS

Due to these findings, it is important that medical personnel be aware of the increase in anterior compartment pressure of the lower leg associated with creatine supplementation. Knowledge of this negative effect is important in diagnosing those individuals more susceptible to lower leg injury, and should allow further identification of any adverse medical conditions that could possibly be prevented. Preventive measures such as stretching, cross-training, slow exercise progression, and avoiding ballistic movements would minimize the exacerbation of present symptoms, and modification of training programs should decrease the possible occurrence of debilitating conditions. Additional study of the effects of creatine supplementation on muscle blood flow, muscle function, and nerve conduction to the anterior compartment of the lower leg appears warranted.

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Creatine deficiency syndromes

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Abstract

Since the first description of a creatine deficiency syndrome, the guanidinoacetate methyltransferase (GAMT) deficiency, in 1994, the two further suspected creatine deficiency syndromes – the creatine transporter (CrT1) defect and the arginine:glycine amidinotransferase (AGAT) deficiency were disclosed.

GAMT and AGAT deficiency have autosomal-recessive traits, whereas the CrT1 defect is a X-linked disorder. All patients reveal developmental delay/regression, mental retardation, and severe disturbance of their expressive and cognitive speech. The common feature of all creatine deficiency syndromes is the severe depletion of creatine/phosphocreatine in the brain. Only the GAMT deficiency is in addition characterized by accumulation of guanidinoacetic acid in brain and body fluids. Guanidinoacetic acid seems to be responsible for intractable seizures and the movement disorder, both exclusively found in GAMT deficiency. Treatment with oral creatine supplementation is in part successful in GAMT and AGAT deficiency, whereas in CrT1 defect it is not able to replenish creatine in the brain. Treatment of combined arginine restriction and ornithine substitution in GAMT deficiency is capable to decrease guanidinoacetic acid permanently and improves the clinical outcome. The lack of the creatine/phosphocreatine signal in the patient's brain by means of *in vivo* proton magnetic resonance spectroscopy is the common finding and the diagnostic clue in all three diseases. In AGAT deficiency guanidinoacetic acid is decreased, whereas creatine in blood was found to be normal. On the other hand the CrT1 defect is characterized by an increased concentration of creatine in blood and urine whereas guanidinoacetic acid concentration is normal.

The increasing number of patients detected very recently suffering from a creatine deficiency syndrome and the unfavorable outcome highlights the need of further attempts in early recognition of affected individuals and in optimizing its treatment. The study of creatine deficiency syndromes and their comparative consideration contributes to the better understanding of the pathophysiological role of creatine and other guanidino compounds in man. (Mol Cell Biochem **244**: 143–150, 2003)

Key words: creatine deficiency, inborn errors of metabolism, GAMT deficiency, AGAT deficiency, creatine transporter deficiency, guanidinoacetic acid

Introduction

The creatine (Cr)/phosphocreatine (PCr) system plays an important role in energy storage and transmission. Beside the mitochondrial and cytosolic creatine kinase (CK) system as a shuttle of high-energy phosphates, synthesis and transport of Cr are integral parts of cellular energy metabolism. Major achievements made over the last couple of years have been attempted to a better understanding of the mechanism of cellular energy homeostasis and their pathophysiological consequences in man. Studies in several animal models (e.g. CK knockout mouse) and on cellular models as well as investigations in patients with inborn errors of energy metabolism (e.g. mitochondrial cytopathies) were undertaken in order to clarify the role of the Cr/PCr and CK systems. Three main proteins are the basis of Cr metabolism, namely arginine:glycine amidinotransferase (AGAT, EC 2.1.4.1), *S*-adenosyl-L-methionine:N-guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2), and the Cr transporter (CrT). In the recent years inborn errors of metabolism have been identified for all three proteins - AGAT deficiency (AGAT-D) [1, 2], GAMT deficiency (GAMT-D [MIM 601240]) [3], and CrT1 defect (CrT1-D [MIM 300036]) [4, 5]. A common feature in all these disorders is the complete lack of Cr/PCr in the brain measured by *in vivo* magnetic resonance spectroscopy (MRS), so that they are subsumed as Creatine Deficiency Syndromes

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(CDS). The clinical presentation of all CDS patients is characterized by developmental delay/arrest, mental retardation, and disturbance of active and comprehensible speech. In GAMT-D guanidinoacetic acid (GAA) is increased in brain, blood, cerebrospinal fluid (CSF), and urine, and seizures refractory to anti-epileptic drugs complicate this disease.

In order to more fully comprehend the clinical presentation, treatment and outcome in the different CDS, it seems important to provide a short introduction into the basics of Cr metabolism in humans (Fig. 1) (for a review see [6]). Cr is either taken up from the food by intestinal absorption, and/or it is synthesized endogenously, primarily in kidney, pancreas, and liver. AGAT catalyzes the reversible transamidination of the guanidino group from arginine to glycine yielding GAA and ornithine. GAMT subsequently catalyzes *S*-adenosyl-L-methionine-dependent methylation of GAA to yield Cr and *S*-adenosyl-L-homocysteine [7]. Cr is then transported through the blood and is taken up into Cr-requiring tissues against a large concentration gradient (plasma [Cr] ~ 50 μM; intracellular [Cr + PCr] up to 40 mM). Uptake into the tissues is afforded by a Na^+- and Cl^--dependent Cr transporter [8]. Cr and PCr are nonenzymatically converted at an almost constant rate (~ 1.7%/day) into creatinine (Crn) which passively diffuses out of the cells and is excreted by the kidneys into the urine. The urinary Crn excretion therefore represents a convenient indicator of the total Cr stores in the body. A 70-kg man contains ~ 120 g Cr, of which > 90% are found in muscle tissue.

The increasing number of recently new recognized patients with CDS opens possibility of comprehensive view on the clinical symptoms, metabolic patterns and treatment outcome in the different disorders. Thus, the study of CDS patients will improve the knowledge of the crucial role of Cr and other guanidino compounds in healthy and disease.

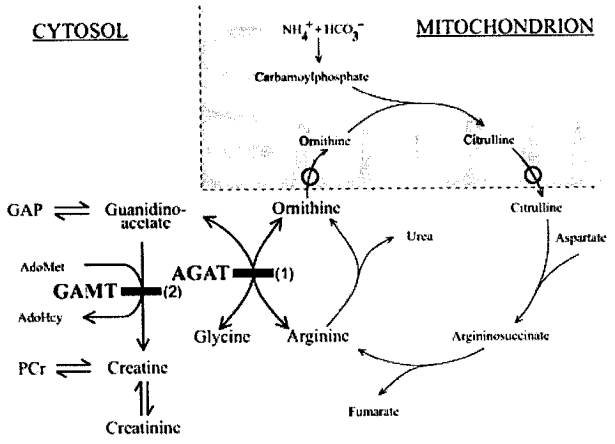

Fig. 1. The metabolic pathway of creatine/phosphocreatine. AdoHcy – S-adenosylhomocysteine; AdoMet – S-adenosylmethionine; AGAT – arginine:glycine amidinotransferase; GAMT – guanidinoacetate methyltransferase; GAP – guanidinoacetophosphate; PCr – phosphocreatine. Sides of metabolic bloc in the two steps of creatine biosynthesis are displayed as filled box and numbered as (1), AGAT deficiency and (2), GAMT deficiency.

Guanidinoacetate methyltransferase deficiency

GAMT-D is the first detected deficiency in creatine biosynthesis in man (for review see [9]).

Enzyme defect

This disorder is caused by deficiency of *S*-adenosyl-L-methionine:N-guanidinoacetate methyltransferase. This enzyme is expressed in liver, kidney, and pancreas [7, 10] and with lower extend also in brain [11], lymphocytes, fibroblasts [12], and other tissues. GAMT catalyzes the *S*-adenosyl-L-methionine-dependent methylation of GAA to yield Cr and *S*-adenosyl-L-homocysteine (Fig. 1). In consequence, GAMT-D leads to a lack of Cr and an accumulation of GAA.

Inheritance and molecular defect

GAMT-D is inherited as an autosomal recessive trait. The GAMT gene mapped to chromosome 19p 13.3 [13]. Four GAMT-D alleles have been characterized so far, 327G → A/309ins13/c.491insG/IVS5-3C → G [12, 14].

Patients

The first patient with GAMT-D was described in 1994 [3]. The clue to its detection was the finding of absent Cr/PCr signal in the brain by *in vivo* proton MRS. Immediately after this first description further patients could be disclosed through verification of missed Cr/PCr signal in the brain by *in vivo* proton MRS [15–22].

Clinical manifestation

The clinical presentation of GAMT-D is heterogeneous. However, in generally it is characterized by developmental delay attracting attention at 6–12 months of age and/or developmental arrest in the second year of life, muscular hypotonia, dyskinetic involuntary movements, no active or comprehensible speech development, severe mental retardation, and seizures in part not controllable by anti-epileptic drugs. In older patients, autism with self-injurious behavior comes along. In patients with a severe phenotype severe extrapyramidal movement disorder and therapy-refractory

epilepsy are predominant, whereas milder affected patients only show developmental delay and mild epilepsy. In some patients abnormalities of brain magnetic resonance imaging (MRI) were described, which consisted in myelination delay or increased signal intensity in T2 weighted images of the globus pallidus.

Diagnostic findings

The pathognomonic laboratory findings in GAMT-D consist of decreased concentration of Cr and Crn and accumulation of GAA. Cr and Crn are lowered in blood and CSF and their excretion in urine is decreased. It is essential to mention, that Crn measurement in blood by the still widely used Jaffé method may lead to misdiagnose the decreased Crn. Verhoeven *et al.* [23] reported that Crn in plasma from 2 GAMT-D patients appeared normal when measured by the Jaffé method but was decreased when measured enzymatically or by HPLC. In urine, the Jaffé method and the enzymatic method gave similar results, indicating that in urine no false elevations of Crn can be expected [23]. For the metabolic urine screening of inborn errors of metabolism the determination of several compounds, e.g. amino acids, organic acids, uric acid, purines, pyrimidines, were usually expressed per mol Crn, because of the relative constancy of the latter (see above). In case of pathological decreased Crn excretion, as in GAMT-D, these compounds might appear to be elevated if expressed per mol Crn. This finding was in fact the clue in the diagnosis of two GAMT-D patients [19, 23]. When generalized elevation of such compounds are found, the possibility of GAMT-D should be considered. GAA, the precursor of Cr, is elevated distinctly in GAMT-D. Increased concentration are found in blood, CSF, and urine. For GAA measurement several methods are used. A simple qualitative test in urine applicable in every metabolic laboratory uses the Sakaguchi reaction [24]. One GAMT-D patient was initially diagnozed by this method [15]. For quantitative results more sophisticated methods, e.g. cation exchange chromatography with pre/post-column derivatization [25] or stable isotope dilution gas chromatography-mass spectrometry [26] can be applied.

The *in vivo* MRS proved as an useful tool in detection of GAMT-D. Cr depletion in brain measured by means of proton MRS is reflected by the absence of the Cr/PCr resonance (Fig. 2). By *in vivo* phosphorus MRS of the brain one can detect the decrease of PCr with concomitant appearance of a usually not detectable resonance assigned to be guanidinoacetophosphate (Fig. 2). Whereas GAMT-D patients reveal a complete lack of the Cr/PCr signal in the brain, the decrease of Cr in their muscle seems not to be so pronounced but even verifiable by MRS (Fig. 3) [15, 20].

For confirmatory diagnosis determination of GAMT activity in liver, fibroblasts, or lymphoblasts is available [27].

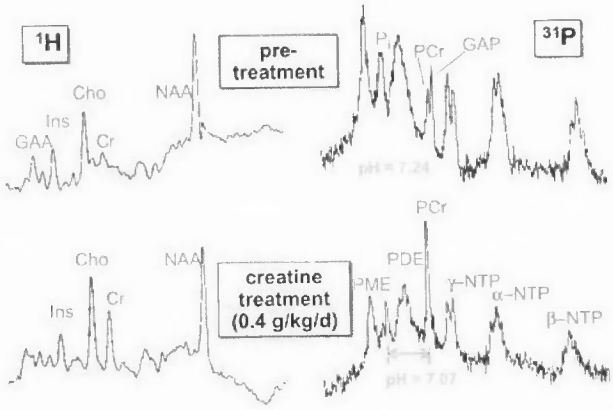

Fig. 2. In vivo proton and phosphorus magnetic resonance spectroscopy of the brain in a patient with guanidinoacetate methyltransferase deficiency [18]. GAA – guanidinoacetic acid; Ins – inositols; Cho – choline-containing compounds; Cr – total creatine (mainly creatine and phosphocreatine); NAA – N-acetyl-L-aspartate; Pi – inorganic phosphate; PCr – phosphocreatine; GAP – guanidinoacetophosphate; PME – phosphomonoester; PDE – phosphodiester; NTP – nucleoside 5′-triphosphate.

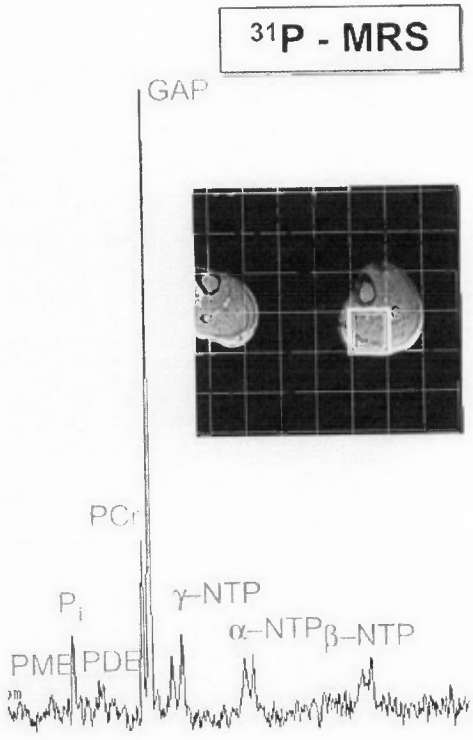

Fig. 3. In vivo phosphorus magnetic resonance spectroscopy of the calve muscle in a patient with guanidinoacetate methyltransferase deficiency [20]. PME – phosphomonoester; Pi – inorganic phosphate; PDE – phosphodiester; PCr – phosphocreatine; GAP – guanidinoacetophosphate; NTP – nucleoside 5′-triphosphate.

Treatment

In GAMT-D treatment approaches attempt to restore depleted creatine in the brain by supplementation of creatine in pharmacological doses. Oral supplementation with 0.35–2.0 g/kg/day of Cr slowly increased the Cr/PCr concentration in the brain. However, even after several months, Cr/PCr in these patients' brain remained significantly below the normal range [9]. Even if Cr replacement also causes decreasing GAA formation, GAA concentration remains largely elevated in CSF, serum and urine [28] which may explain the persistence of some of the clinical symptoms, especially epilepsy. Dietary arginine restriction (15 mg/kg/day) in combination with ornithine supplementation (100 mg/kg/day) led to a substantial and permanent decrease of GAA in body fluids of one patient. Biochemical effects were accompanied by a marked clinical improvement. Distinctly reduced epileptogenic activities in electroencephalography accompanied by almost completely disappearance of seizures demonstrates the positive effect of GAA reduction, indicating for the first time that GAA may exert an important epileptogenic potential in man [28].

Outcome

All patients benefited from Cr supplementation, although to different degrees; however, none has returned to a normal developmental level, and all patients still lack active speech. In the first months after initiating Cr treatment an improvement affecting dyskinesia, muscular hypotonia, seizures, alertness, social contact, and behavior is ascertainable. However, thereafter delay in further clinical improvement becomes evident. Especially in patients with the severe phenotype their clinical circumstances may deteriorate and therapy-refractory seizures reoccur. This clinical course is timely correlated with the observed changes of Cr/PCr in the brain. Cr/PCr concentration increases during the first months of treatment reaching only ~ 50% of controls after some years of treatment [9]. Beside the inability to fully correct the Cr/PCr deficit in brain the neurotoxic action of GAA can contribute to the unsatisfying clinical course. The impact of a combined approach of Cr supplementation and GAA lowering still has to be elucidated.

X-linked creatine transporter deficiency

The CrT1-D is the second CDS which was disclosed [4, 5].

Protein defect

CrT1-D is caused by a defective CrT1. The CrT1 gene is expressed in most tissues, with highest levels in skeletal muscle and kidney and somewhat lower levels in colon, brain, heart, testis, and prostate [29, 30]. *In situ* hybridization studies in adult rat brain revealed the presence of the CrT1 in neurons and oligodendrocytes, but not in astrocytes [11]. A second Cr transporter, CrT2, is expressed in testis only. The Cr transporters are members of solute-carrier family 6 (neurotransmitter transporters). Cr, mainly synthesized in the liver, is transported through the blood and is taken up into Cr-requiring tissues against a large concentration gradient. Uptake into the tissues is afforded by a Na^+- and Cl^--dependent Cr transporter.

Inheritance and molecular defect

CrT1-D is an X-linked disorder. The CrT1 gene, now called 'SLC6A8' (MIM 300036), has been mapped to chromosome Xq28 [31]. One CrT1-D allele, 1539C → T, causing a hemizygous nonsense mutation has been reported so far [5].

Patients

The first patient, a 6 years old boy, has been described recently [4]. Furthermore, two brothers of an independent family were detected in the same institution (Neurological Division at Children's Hospital Medical Center in Cincinnati, USA) [32, 33]. Very recently in three brothers of an other family the CrT1-D could been disclosed [33].

Clinical manifestation

The index patient initially presents with mild mental retardation, mild epilepsy, but with severe delay both in speech and in expressive-language function. In addition, mild central hypotonia was observed, but gross and fine motor functions were normal. In three female relatives of the index patient, mild biochemical abnormalities and learning disabilities were reported [4]. In all three male patients from Cincinnati common findings were developmental delay, mild epilepsy, but expressive dysphasia. None of the patients has muscle or heart problems. Increasing behavior problems and development of brain atrophy in adolescence points to a slowly progressive disorder [32].

Diagnostic findings

In CrT1-D the Cr concentration both in plasma and urine is elevated. Crn in plasma was found to be normal. In contrast to GAMT-D the GAA concentration in plasma and urine is normal. *In vivo* proton MRS revealed the complete lack of

the Cr/PCr signal in the brain. Confirmatory diagnosis can be made by a recently developed investigation of Cr uptake in fibroblasts, by which it is possible to discriminate between patients, carriers, and controls [5].

Treatment

Treatment of the index patient with oral Cr monohydrate (0.34 g/kg/day) for 3 months resulted in increased Cr concentration in CSF and urine. However, the follow-up proton MRS performed 4 months later demonstrated a similar absence of Cr/PCr as seen before treatment. Due to the fact that the clinical symptoms even did not improve, Cr treatment does not appear to be useful. The oral substitution of Cr was discontinued.

Outcome

Since there is no effective treatment, the course of the slowly progressive neurological syndrome with mental retardation, sever speech impairment and progressive atrophy of the brain can not be influenced so far.

Arginine:glycine amidinotransferase deficiency

AGAT-D, at first reported as 'reversible brain Cr deficiency' in two sisters [1], is the third CDS which could be disclosed [2].

Enzyme defect

AGAT-D is caused by deficiency of L-arginine:glycine amidinotransferase (EC 2.1.4.1), which catalyzes the first and rate-limiting step in creatine biosynthesis, which is the reversible transamidination of the guanidino group from arginine to glycine to yield GAA and ornithine (Fig. 1). Immunoreactive enzyme was proven with highest content in the proximal tubules of the kidney, but also in hepatocytes and in alpha cells of the pancreas of the rat [10]. *In situ* hybridization studies in adult rat brain revealed an ubiquitous neuronal and glial expression of AGAT [11].

Inheritance and molecular defect

AGAT-D is an autosomal recessive disorder. The AGAT gene has been mapped to chromosome 15q11.2. One AGAT-D allele, 9279G → A, homozygously causing a truncated protein has been reported so far [2].

Patients

Two sisters of unrelated Italian parents have been detected so far [1].

Clinical manifestation

The two sisters, 4 and 6 years of age, suffered from mild mental retardation and severe language delay. They started walking unaided at 24 months and started speaking the first words at 30 months. Beside one uncomplicated febrile seizure in one girl, they had no further seizures. Their brain magnetic resonance imaging were normal. Both of them never expressed muscular or other neurological symptoms.

Diagnostic findings

Cr in serum as well as the amino acids arginine and glycine was reported to be normal. The GAA concentration in serum was slightly decreased, whereas the excretion of GAA in urine was found to be extremely low. *In vivo* proton MRS revealed the total absence of Cr/PCr in the brain, thus suspecting a CDS. The underlying enzyme defect could be established by undetectable AGAT activity, as investigated radiochemically in fibroblasts and lymphoblasts [2].

Treatment

Cr supplementation at a rate of 400 mg/kg/day increased Cr/PCr in the brain to 40 and 80% of controls within 3 and 9 months, respectively. After 16 months of treatment a nearly complete replenishment of the Cr/PCr signal has been reported.

Outcome

Cr supplementation led to a rapid progress in the acquisition of visual perceptual and fine motor skills, together with a slower rate of general cognitive development in the younger sister, but not in the elderly. Language abilities also improved, but more slowly than nonverbal skills.

Comparative consideration of creatine deficiency syndromes

The summarized comparison of the clinical features in the different CDS is shown in Table 1.

Table 1. Comparative findings in creatine deficiency syndromes

	GAMT deficiency	CrT1 deficiency	AGAT deficiency
Patients	10 (7 published, incl. 1 in abstr./3 unpublished) *1	6 (3 published, incl. 2 in abstr./3 unpublished)*2	2 (2 published)*3
Gender	1 female/8 male	3 male	2 female
Origin	Kurdish/German/Welsh/2 Turkish/Italian	Caucasian	2 Italian
Consanguinity	2 consang./4 unrelated	?	None
Age at onset	Developm. delay median 5 mo (3–7 mo) seizures median 2½ yrs (10 mo–4 yrs)	7 mo in 1/3	?
Age at diagnosis	Median 3 yrs 8 mo (19 mo–26 yrs)	6, 16, 20 yrs	4 yrs 4 mo/6 yrs 5 mo
Developmental delay/arrest	8/8	mild 3/3	2/2
Hypotonia	7/8	1/3	0/2
Dyskinesia	4/8	0/3	0/2
Reflexes, increased	2/7	0/3	0/2
Seizures	7/8 untractable seizures: 5/8	mild 3/3	None (only one febrile seizure)
Mental retardation	Severe 7/8, mild 1/8	mild 3/3	Severe 2/2
Autism/self-injurious behaviour	7/7	1/3	0/2
Active speech	None 7/8, single words 1/8	Single words, severe expressive dysphasia 3/3	Delayed 2/2
MRI	Myelination delay 3/8, T2 intens Pallidum 3/8 (1 pat. with both), no MRI changes 3/8	White matter lesion 2/3 Brain atrophia 2/3	Normal
EEG (pathol)	7/7	1/3	?
Treatment	Creatine 350–1250 (–2000) mg/kg/day 8/8 Arginine restrictive diet 3/8 Phenylbutyrate 1/8 Sodium benzoate 1/8	None	Creatine 400 mg/kg/day 2/2
Outcome	Unfavourable		Satisfying
Motor development	Improved		Improved 1/2
Active speech	None		Improved 1/2
Social contact/behaviour	Improved	No changes	Improved 1/2
Seizures	Improved (after GAA lowering)		
Mental retardation	Severe		

*1 references [3, 12, 14–22, 28, 35]; *2 references [4, 5, 32, 33, 36]; *3 references [1, 2].

Conclusion

The different Cr metabolism defects are highly instructive, thus allowing interesting conclusions, but also raise new challenging questions.

The GAMT-D revealed the most severe phenotype. Extrapyramidal symptoms and intractable seizures were exclusively present in this disease. In addition, autistic, self-injurious behavior seems to be characteristic for GAMT-D. Clinical symptoms only found in GAMT-D might be attributed to the influence of GAA, which is highly increased in this disease. Furthermore, the combined impact of Cr defi-

ciency and GAA accumulation might be responsible for the most severe phenotype.

Developmental delay/arrest, mental retardation, even if in different degrees, and impairment of active and cognitive speech are common findings in all three disorders. This points to the special effects caused by Cr deficiency.

Mildest clinical symptoms were found in AGAT-D. The finding of a normal serum concentration of Cr in the two sisters with AGAT-D indicates that they efficiently take up dietary Cr (which in adults on average contributes ~ 50% to the daily Cr requirements) into blood and most likely also into muscle. The complete lack of the Cr/PCr signal in MRS in their brain despite of normal plasma Cr concentration suggests that the synthesis of Cr is also affected within the brain. Assuming that the normal brain is self-sufficient in terms of Cr biosynthesis, one would expect a more severe phenotype in AGAT-D than in CrT1-D, what in fact could not be established in the patients reported so far. The lack of Cr in the brain of CrT1-D patients and their severe phenotype reveals some doubts about the brain's capacity for de novo Cr biosynthesis. Similar to the rest of the body where the liver is the primary site of Cr biosynthesis, even containing only low levels of Cr itself, there may be a strict separation of Cr-synthesizing and Cr-accumulating cells in the brain as well. If this is the case, the Cr transporter would be required for significant accumulation of Cr in the brain.

The fact that normal concentrations of Cr in serum in AGAT-D or even supranormal concentrations by supplementation with rather high doses of Cr in GAMT-D failed to normalize brain Cr/PCr points to the impact of a limited permeability of the blood-brain barrier for Cr. Beside other findings, this is further corroborated by the observation that in GAMT-D patients ingesting Cr, Cr uptake into the brain is a very slow process and steady-state concentrations still below the normal range were only reached after several months. Why in both AGAT-D patients the Cr replenishment was nearly completely after 16 months of treatment and if there is an compromising impact of GAA on the Cr uptake in the brain has to be established in the future.

The therapeutic outcome in CDS is different. Whereas in CrT1-D there exists no therapeutic option so far, the preliminary results of therapeutic attempts in AGAT-D seems to be promising. Despite of partial improvement by Cr supplementation the outcome in GAMT-D is still unfavorable. Even GAA lowering attempts by arginine restriction and ornithine substitution revealed distinct additional effects on the clinical course, it was not possible to normalize general condition. Further therapeutic attempts must be focussed on alternative modes of Cr application and on additional approaches to lower the GAA concentrations.

The early detection of affected patients and the timely onset of treatment might also improve the outcome especially in GAMT-D and AGAT-D. The detection of GAMT-D already in the newborn period is feasible. In one patient increased GAA was proven in the retrospective analyzed dried blood spot specimen kept from neonatal screening [34]. This points to the usefulness of a neonatal screening of GAMT-D by measurement of GAA. However, as our preliminary results with such an screening by means of tandem mass spectrometry have shown, we did not find any affected child out of more than 150.000 neonates (unpublished observation). Nevertheless, at least in all patients with developmental delay and speech impairment of unknown origin, and particularly if accompanied by an extrapyramidal movement disorder and/or seizures, a CDS should be excluded carefully.

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REVIEW

DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis

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The heterocyclic amines (HCAs) are a family of mutagenic/carcinogenic compounds produced during the pyrolysis of creatine, amino acids and proteins. The major subclass of HCAs found in the human diet comprise the aminoimidazoazaarenes (AIAs) 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). All, except DiMeIQx, have been shown to be carcinogenic in animals. These compounds are present in cooked muscle meats at the p.p.b. level. Since the discovery of the HCAs in the late 1970s, many studies have examined the DNA adducts of these compounds. This review compiles the literature on AIA–DNA adducts including their identification and characterization, pathways of formation, mutagenesis *in vitro* and *in vivo*, and their association with carcinogenesis in animal models. It is now known that metabolic activation leading to the formation of DNA adducts is critical for mutagenicity and carcinogenicity of these compounds. All of the AIAs studied adduct to the guanine base, the major adduct being formed at the C8 position. Two AIAs, IQ and MeIQx, also form minor adducts at the N^2 position of guanine. A growing body of literature has reported on the mutation spectra induced by AIA–guanine adducts. Studies of animal tumors induced by AIAs have begun to relate AIA–DNA adduct-induced mutagenic events with the mutations found in critical genes associated with oncogenesis. Several studies have demonstrated the feasibility of chemoprevention of AIA tumorigenesis. Only a few studies have reported on the detection of AIA–DNA adducts in human tissues; difficulties persist in the routine detection of AIA–DNA adducts in humans for the purpose of biomonitoring of exposure to AIAs. The AIAs are nevertheless regarded as possible human carcinogens, and future research on AIA–DNA adducts is likely to help address the role of AIAs in human cancer.

Abbreviations: AIA, aminoimidazoazaarene; AMS, accelerator mass spectrometry; BcPHDE, benzo[*c*]phenanthrene diol epoxide; CHL, chlorophyllin; CLA, conjugated linoleic acid; DiMeIQx, 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline; dG-C8–IQ, *N*-(deoxyguanosin-8-yl)–IQ; dG-C8–MeIQ, *N*-(deoxyguanosin-8-yl)–MeIQ; dG-C8–MeIQx, *N*-(deoxyguanosin-8-yl)–MeIQx; dG-N^2–MeIQx, 5′-(deoxyguanosin-N^2-yl)–MeIQx; dG-C8-4,8–DiMeIQx, *N*-(deoxyguanosin-8-yl)-4,8–DiMeIQx; dG-C8–PhIP, *N*-(deoxyguanosin-8-yl)–PhIP; dG-N^2–IQ, 5′-(deoxyguanosin-N^2-yl)–IQ; GC–MS, gas chromatography–mass spectrometry; HCA, heterocyclic amine; I3C, indole-3-carbinol; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; NAT, *N*-acetyltransferase; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; WBC, white blood cell.

Introduction

Twenty years ago the discovery that cooked fish and beef showed highly mutagenic activity, as detected by the Ames/*Salmonella* test system (1,2), began an intensive search for the mutagens present in these foods. A number of studies showed that these mutagens were formed during the pyrolysis of amino acids and proteins, and during the cooking of a variety of muscle meats (1–6). The novel mutagens were identified as heterocyclic amines (HCAs). The major subclass of HCAs found in cooked meats was identified as the aminoimidazoazaarenes (AIAs), which includes those compounds with a quinoline, quinoxaline or pyridine moiety.

Studies to date indicate that the AIAs are present at the p.p.b. (ng/g) range in meats cooked by ordinary household methods (3–7). The formation of these compounds in meats depends largely on cooking temperature and duration (3,4,8,9). Chemical modeling has shown that several of the AIAs are produced by the reaction of creatinine and free amino acids at normal cooking temperatures (reviewed in refs 4,10). These compounds are distinct from the polycyclic aromatic hydrocarbons derived from the pyrolysis of fat in meat, which occurs, for example, during barbecuing. In addition to their mutagenic activity in *in vitro* assays, 10 HCAs bioassayed for carcinogenicity to date have all been shown to be carcinogenic in rats and/or mice (11,12); one of the AIAs, IQ, has also been shown to be carcinogenic in monkeys (13). The carcinogenicity of the AIAs most ubiquitously present in cooked beef (14) is summarized in Table I.

The HCAs, like the majority of chemical mutagens/carcinogens, form DNA adducts. It is generally accepted that DNA adducts of chemical carcinogens play a role in carcinogenesis; however, the relationship between DNA adducts and carcinogenesis is not fully understood (31). Since the identification of HCAs in cooked meats, considerable progress has been made in characterizing HCA–DNA adducts and clarifying pathways of metabolism necessary for DNA adduct formation. In addition, many investigations have attempted to relate HCA–DNA adduct formation to mutagenic and carcinogenic activity. In light of the considerable expansion of HCA research over the recent years, and the importance of increasing our understanding of the influence of DNA adducts in carcinogenesis, the current review examines HCA–DNA adducts and their possible role in carcinogenesis. Specific topics addressed in this review include (i) the characterization and identification of HCA–DNA adducts; (ii) the relationship between HCA–DNA adducts and mutagenic events; and (iii) the formation of DNA adducts in animal models as it relates to carcinogenesis. We have focused this review on the

Table I. Carcinogenicity of heterocyclic amines

Compound	Strain/species	Target organs	Reference
IQ	F344 rat	Liver, small and large intestine, Zymbal gland, clitoral gland, skin	15
	Sprague–Dawley rat	Liver, mammary gland, Zymbal gland	16
	CDF_1 mouse	Liver, forestomach, lung	17
	$B6C3F_1$ mouse	Liver	18
	Monkey	Liver	13
MeIQ	F344 rat	Large intestine, Zymbal gland, skin, oral cavity, mammary gland	19
	CDF_1 mouse	Liver, forestomach	20
MeIQx	F344 rat	Liver, Zymbal gland, clitoral gland, skin	21,22
	CDF_1 mouse	Liver, lung, hematopoietic system	23
	$B6C3F_1$ mouse	Liver	18
PhIP	F344 rat	Large intestine, prostate, lymphoid tissue (males), mammary gland, large intestine (females)	24,25,26 24,27
	Sprague–Dawley rat	Mammary gland	28
	CD rat	Mammary gland	29
	CDF_1 mouse	Lymphoid tissue	30
	$B6C3F_1$ mouse	Liver	18

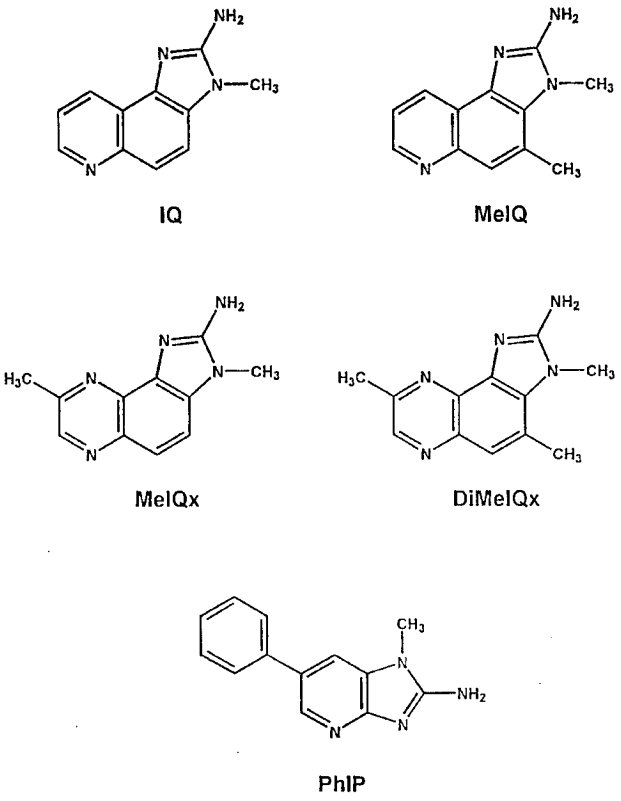

Fig. 1. Chemical structures of several of the AIA-type HCAs.

AIA-type HCAs (Figure 1), which are most consistently found in cooked meats and most prevalent in the human diet (14).

Metabolic activation and AIA–DNA adduct formation

The AIAs are promutagens/pro-carcinogens that require metabolic activation for DNA adduct formation (32–71). The major pathway of AIA activation involves phase I hepatic cytochrome P450-mediated *N*-hydroxylation followed by phase II esterification of the *N*-hydroxylamines to reactive ester derivatives that covalently modify DNA. Numerous studies using inducers and inhibitors of cytochromes P450, antibodies to cytochromes P450, purified cytochromes P450 and recombinant enzymes have identified CYP1A2 as having high specificity and catalytic activity for AIA *N*-hydroxylation (33–67). Other cytochromes P450, including CYP1A1, CYP3A4, CYP2C9/10, CYP2A3 and CYP1B1, also carry out AIA *N*-hydroxylation, but these cytochromes generally show a lower capacity toward *N*-hydroxylation than CYP1A2 (46,48–54,57,59,61–63,68–71).

The *N*-hydroxylamine metabolites of the AIAs react relatively poorly with nucleic acids and require a second metabolic activation step to highly reactive ester derivatives to facilitate AIA–DNA adduct formation. To date, four mammalian phase II cytosolic enzymes have been implicated in the metabolic activation of the *N*-hydroxy-AIAs: *N*-acetyltransferase (NAT), sulfotransferase, prolyl tRNA synthetase and phosphorylase (35,36,57,58,67,72–81). These enzymes produce *N*-acetoxy, *N*-sulfonyloxy, *N*-prolyloxy and *N*-phosphatyl ester derivatives, respectively, at the exocyclic amino group (Figure 2). Many studies have now demonstrated that AIA–DNA adduct formation is greatly enhanced by phase II esterification of the *N*-hydroxylamine derivatives (35,36,67,72,74–82). For example, in an *in vitro* assay, NAT-mediated metabolism of *N*-hydroxy-IQ to *N*-acetoxy-IQ produced at least a 30-fold increase in IQ–DNA adduct levels (72). The four phase II activation pathways appear to give rise to the same AIA–DNA adduct(s) (76).

Several studies support the concept that the levels of AIA–DNA adducts formed in various tissues *in vivo* will largely depend on the balance between metabolic activation and detoxification (79,83–90). The liver is the major site of AIA metabolism *in vivo*, and hepatic phase I activation capacity, determined in large part by hepatic CYP1A2 levels, has a major impact on hepatic and as well as extrahepatic AIA–DNA adduct levels. For example, studies in monkeys and rats indicate that AIA–DNA adduct formation in liver and extrahepatic tissues is influenced by hepatic cytochrome P450-mediated AIA *N*-hydroxylation (79,83,84). The low level of MeIQx–DNA adducts in tissues of cynomolgus monkeys given MeIQx correlates with a low capacity for hepatic cytochrome P450-mediated *N*-hydroxylation of MeIQx in this species (63,83,84). In rats given PhIP, PhIP–DNA adducts are detected in colon despite the apparent lack of phase I activation of PhIP in colon (79). Recently, a breakdown product of *N*-acetoxy–PhIP was found in blood of rats given PhIP (79), which supports the idea that reactive metabolites, produced

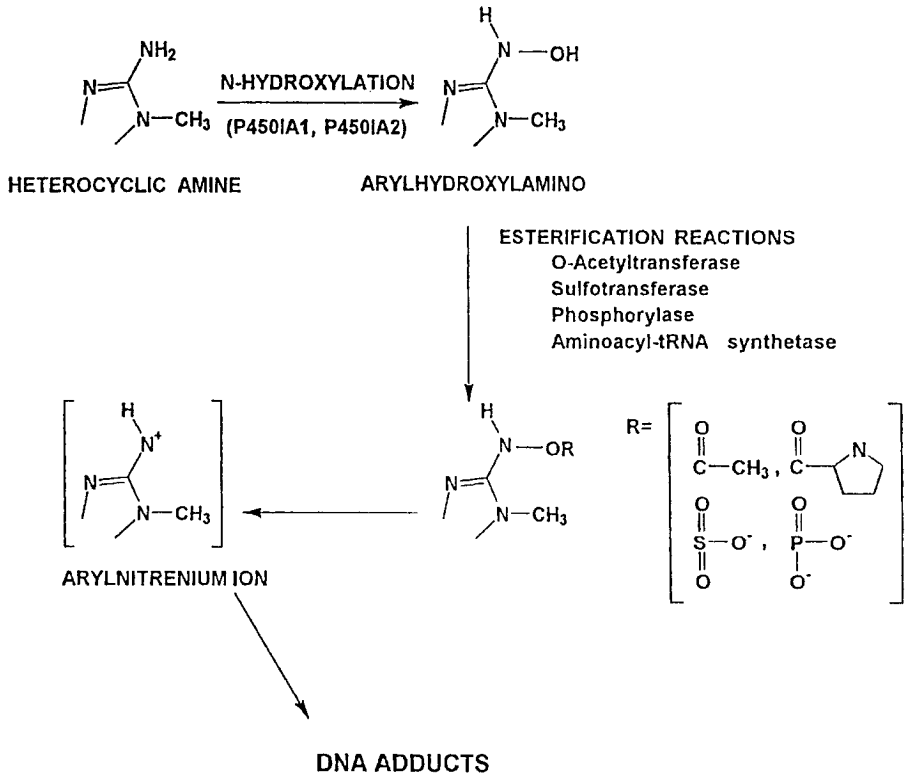

Fig. 2. Bioactivation of heterocyclic amines. Pathways of AIA metabolic activation leading to DNA adduct formation.

largely by hepatic metabolism, circulate via the bloodstream contributing to PhIP–DNA adducts in extrahepatic tissues such as colon (79) and mammary gland (90). In light of studies showing detectable AIA phase I metabolic activation by microsomes or cultured cells from several extrahepatic tissues, such as the lung, kidney, mammary gland and possibly pancreas (91–99), it is also possible that *in situ* *N*-hydroxylation will contribute to the formation of AIA–DNA adducts in several extrahepatic sites. In addition, DNA adducts in extrahepatic tissues might also arise from the metabolic activation of AIAs via prostaglandin H-synthetase (100–103). Recently the peroxygenase activity of cytochrome P450 has been shown to activate IQ to a reactive metabolite that binds to 2′-deoxyguanosine (104,105). The contribution of this pathway to tissue AIA–DNA adduct levels *in vivo*, however, is not yet known.

Extrahepatic phase II activity may also influence extrahepatic DNA adduct formation by contributing to the further metabolic activation or detoxification of the *N*-hydroxylamines or *N*-acetoxy metabolites derived from the circulation or generated *in situ* (79,88,90,106,107). In contrast to the comparatively low cytochrome P450-mediated activation capacity in extrahepatic tissue versus liver, phase II esterification activity in extrahepatic tissues is often similar to, or higher than, the activity found in liver (76,79,90). For example, in female rats, cytosolic NAT activation of *N*-hydroxy–PhIP was reported to be 16-fold higher in the mammary gland than in the liver (90). This difference in NAT activity may, in part, contribute to the 10-fold higher PhIP–DNA adduct levels in mammary gland than in liver in these animals (90,108).

The esters of *N*-hydroxy–AIAs generated by phase II metabolism are transient metabolites that react with nucleophilic sites in DNA. The ester moieties serve as leaving groups giving rise to putative electrophilic arylnitrenium ion intermediates (Figure 2) considered for many years to be involved in arylamine DNA adduct formation (109). Although to a much lesser extent, arylnitrenium ions may also be generated directly from the *N*-hydroxylamine metabolite following the protonation of the *N*-hydroxylamino group (109,110). This reaction mechanism explains the DNA adduction of certain *N*-hydroxy–AIAs, such as *N*-hydroxy–IQ, in the absence of esterification (110). The arylnitrenium ion is generally considered to be the ultimate carcinogenic form of the AIAs responsible for the formation of AIA–DNA adducts (109–115). The reactivity of the arylnitrenium ions and their carbenium ion resonance forms with particular nucleophilic sites on DNA give rise to specific DNA adducts, and with the AIAs, adducts have been found at the C8 and N^2 positions of guanine (110,116).

Identification of AIA–DNA adducts

Several studies have examined AIA–DNA adduct formation by various methods, including the ^{32}P-post-labeling assay (110–143). The structures of the various AIA–DNA adducts identified to date are shown in Figure 3. All form a major adduct with the C8 atom of guanine and, for IQ and MeIQx, a minor adduct with the N^2 atom of guanine has also been identified (110,116–122,128,129,134–138). *N*-acetoxy derivatives of several AIAs have also been shown to react with other bases *in vitro* (120,129,138), but such reactions appear to be of no quantitative importance *in vivo*. Studies that identified AIA adducts *in vitro* and *in vivo* are summarized in Table II.

Although each AIA forms one major guanine adduct, ^{32}P-post-labeling analysis (130) has shown that most AIAs yield

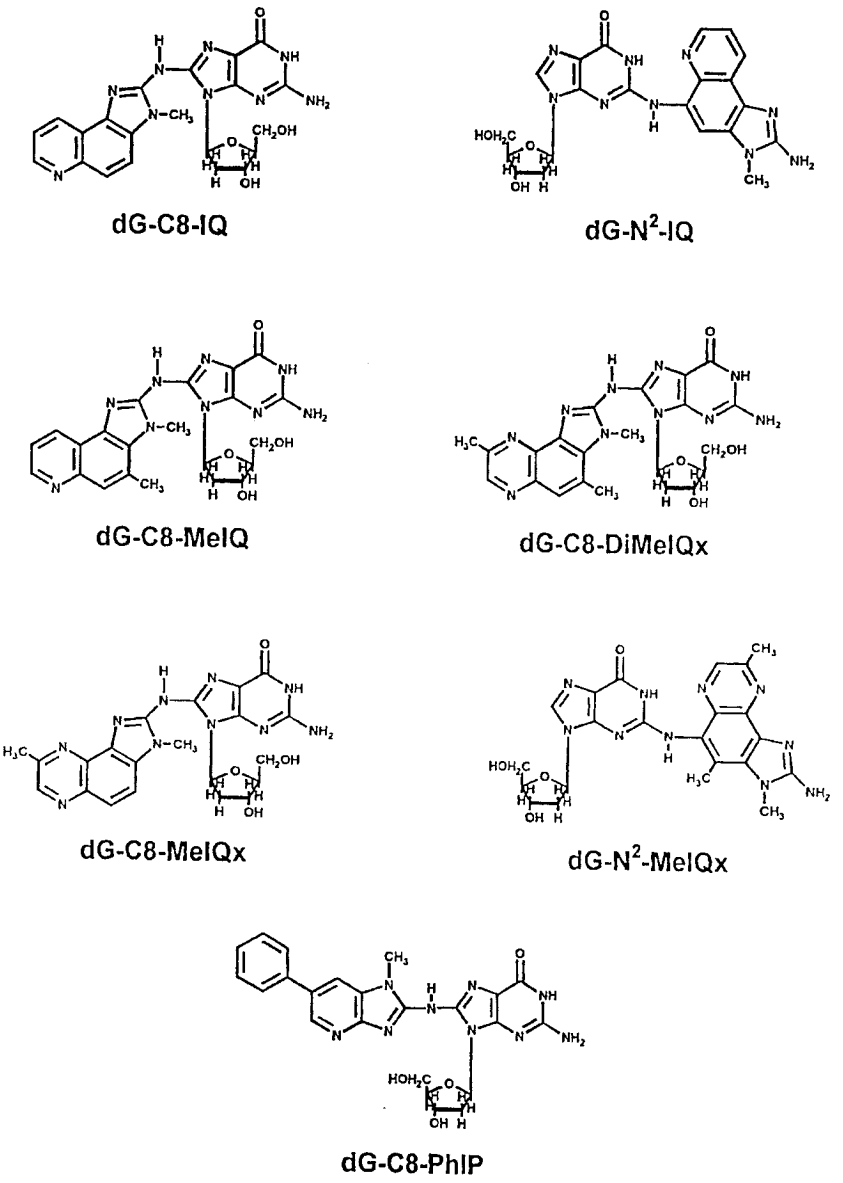

Fig. 3. Structures of the AIA–DNA adducts characterized to date. dG-C8–IQ, *N*-(deoxyguanosin-8-yl)-IQ; dG-N^2–IQ, 5′-(deoxyguanosin-*N*^{2}-yl)-IQ; dG-C8–MeIQ, *N*-(deoxyguanosin-8-yl)–MeIQ; dG-C8-4,8–DiMeIQx, *N*-(deoxyguanosin-8-yl)-4,8–DiMeIQx; dG-C8–MeIQx, *N*-(deoxyguanosin-8-yl)–MeIQx; dG-*N*^{2}–MeIQx, 5′-(deoxyguanosin-*N*^{2}-yl)–MeIQx; dG-C8–PhIP, *N*-(deoxyguanosin-8-yl)–PhIP.

multiple DNA adduct spots, especially in DNA from animals treated with AIAs (117,118,120–129,131–133,139–143). Several studies have indicated, however, that incomplete digestion of DNA to 3′-monophosphonucleotides prior to 5′ ^{32}P-labeling may result in radiolabeled dimers and/or higher oligomers each containing the major adduct. Additional digestion procedures using nuclease P1 alone or with phosphodiesterase has been shown to reduce the multiple adducts observed with several HCAs to essentially one major adduct (128,129,142,143).

While the ^{32}P-labeled adduct patterns of a particular AIA may differ greatly among various laboratories, the major identified DNA adduct for each AIA is similar in all species examined, including bacteria (*Salmonella*), rats, mice and monkeys (117–119,120–122,128,129,134–137,140). Examination of reaction products formed *in vitro* between *N*-acetoxy–PhIP and calf thymus DNA by fluorescence spectroscopy indicated the presence of four adduct fluorophores with different conformations (144). The data suggested heterogeneous formation of PhIP–DNA adducts in intact DNA but it could not be distinguished whether PhIP formed multiple adducts or if a given adduct existed in multiple conformations.

Genotoxicity of HCA–DNA adducts in vitro

Mutagenicity in bacterial assays

All the AIAs, with the exception of PhIP, are extremely potent mutagens in the Ames assay with GC frame-shift sensitive tester strains *Salmonella* TA98 and TA1538 (145,146). Mutagenicity of the AIAs in these bacterial strains is in the order of MeIQ > IQ > DiMeIQx ≥ MeIQx > PhIP (3). Many studies in bacterial assays support the role of AIA–DNA

Table II. References for studies reporting the identification and detection of AIA–DNA adducts

Adduct	Characterization[a]	Detection *in vitro*[b]	Detection *in vivo*[c]
dG-C8-IQ	110,116	110,116	117–122
dG-N^2-IQ	116	116,119	119,121,122
dG-C8-MeIQ	128	–	128
dG-C8-MeIQx	116,129	116,129	116,120,129
dG-N^2-MeIQx	116	116	–
dG-C8-DiMeIQx	134	134	134,135
dG-C8-PhIP	136–138	136–138	136,137

[a]Adduct characterization is routinely carried out by nuclear magnetic resonance, mass and ultraviolet spectroscopy.
[b]AIA adducts are frequently produced *in vitro* by reacting *N*-acetoxy-AIA with calf thymus DNA or with deoxyguanosine. Prior to characterization, AIA adducts are purified from enzymatically hydrolyzed DNA or from the deoxyguanosine reaction mixture by high performance liquid chromatography (HPLC).
[c]AIA–DNA adducts have been detected *in vivo* by ^{32}P-post-labeling or HPLC analysis of DNA from animals treated with AIAs.

adduct formation in mutagenesis (3,4,145–156). For example, in studying the relationship between PhIP–DNA adduct levels and mutagenicity, Malfatti *et al.* (149) determined that ~25 adducts are required for one mutational event in *Salmonella* TA98. In addition, Kerdar *et al.* (148) showed that there was a linear relationship between DNA binding and mutagenicity among several AIAs: the more potent the mutagen, the higher the level of adducts in bacterial DNA. Therefore, the difference in mutagenicity among the AIAs is largely attributable to quantitative differences in DNA adduct levels rather than to qualitative differences between AIA adducts.

In *Salmonella* strain TA1538, IQ, MeIQ and PhIP caused a single GC deletion in a run of GCs in the *hisD* gene, which is consistent with the known preference for AIA–DNA adduct formation at guanine (146,157). In addition, in the base substitution *Salmonella* strain TA100, PhIP induced predominantly GC→TA transversion; error-prone bypass of the dG-C8–PhIP adduct appeared to be associated with the induction of this mutation (156). Consistent with the lack of formation of adenine adducts, AIAs were poor mutagens in *Salmonella* strains specific for AT frameshift changes (3). AIA mutagenicity studies in other bacterial genes such as *lacZ*, *lacZa* and *lacI* of *Escherichia coli* further showed that mutations occur primarily at G:C pairs; however, the types of guanine mutations (base substitution or frameshift) were different among the various assays (158–160).

Genotoxicity and mutagenicity in mammalian cells

The genotoxicity of the AIAs in cultured mammalian cells has been assessed by multiple end points including mutagenesis, chromosomal aberrations, sister-chromatid exchange, DNA repair synthesis and DNA strand breaks (34,55,161–181). Whereas AIAs are potent frameshift mutagens in *Salmonella* bacterial reversion assays, in mammalian cell mutagenicity assays, AIA–DNA adducts induce primarily base substitution mutations at guanine (175–177,180). PhIP- and/or IQ-induced mutations have been examined in the *hprt*, *dhfr* and *aprt* genes of Chinese hamster ovary cells, and in the *hprt* gene of human lymphoblastoid cells (175–177,180). For both compounds, G→T transversion mutations in guanine nucleotides adjacent to either guanine or adenine nucleotides occurred most frequently. In the *supF* shuttle vector mutagenicity assay, ~25% of the mutations induced by PhIP in repair-proficient mammalian cells were at guanine in the sequence 5′-GCAGA-3′ (174). In the *dhfr* gene of Chinese hamster ovary cells treated with PhIP, recurring guanine mutations were also observed at CAGG, and one of 20 characterized mutants harbored a mutation in the sequence CAGA (175). AIA-induced frameshift mutations found in mammalian cells also largely involved a deletion of a guanine base adjacent to guanine and/or adenine (175,176,180). Yadollahi-Farsani *et al.* (180) reported finding a –1 frameshift mutation (guanine deletion) in a 5′-GGGA-3′ sequence in the *hprt* gene of Chinese hamster ovary cells exposed to PhIP that accounted for four of seven frameshift mutations observed in their study.

The role of AIA–DNA adducts in mutagenesis was explored in the *supF* shuttle vector replicated in mammalian cells (161,174). Mutation frequency increased linearly with IQ and PhIP–DNA adduct levels in the vector. ^{32}P-post-labeling analysis showed that IQ and PhIP formed adducts only at guanine nucleotides in the *supF* plasmid, with the C8–guanine adduct being the major lesion (174). Using a polymerase arrest assay, these guanine adducts were shown to be distributed non-randomly in the *supF* gene with the concentration of adducts being different at different guanine sites. Although mutations were found at sites where IQ– and PhIP–guanine adducts formed, adduct level did not correlate with the likelihood of a mutation at a particular site. Therefore, the frequency of a mutation at a particular guanine nucleotide in the *supF* gene appeared to be attributable to other factors besides adduct levels. A similar observation was made in studies concerning MeIQx–DNA adducts in the *E.coli lacZa* gene (160).

In summary, the *in vitro* mutagenicity studies to date point to preferred sites for AIA–DNA adduct-driven mutations in mammalian genes. In support for the role of AIA adducts in G→T transversion mutations, a site-specific mutagenesis study recently reported that the C8-guanine adduct of PhIP induced this mutation (182). It is anticipated that the results from *in vitro* mutagenicity studies will provide insight into the role of AIA–DNA adducts in mutations found in genes associated with AIA-induced carcinogenesis.

Genotoxicity and mutagenicity of AIA–DNA adducts in vivo

Cytogenetic assays, including chromosome aberrations, micronucleated normochromatic erythrocytes and sister chromatid exchanges of bone marrow and peripheral blood of rodents dosed with AIAs, indicate that the AIAs and, by extension, AIA–DNA adducts, appear to be only weakly clastogenic *in vivo* (183–187). In contrast, AIAs have been shown to induce base substitution and/or deletion mutations *in vivo* in the *lacI* or *lacZ* mutational reporter gene of transgenic mice (187–193), the colonic crypt cells of mice (194–196), *Dlb-1*-specific locus test in mouse small intestine (191,197,198) and the animal-mediated microbial assay system (199).

In *lacI* and *lacZ* transgenic mice, mutant frequency appears to depend on the dose and the duration of exposure to AIAs suggesting that the higher the level of adducts is and the more persistent the adducts are, the greater is the mutagenic effect in these mice (187,190,191). The role of persistent AIA adducts in mutagenesis *in vivo* is illustrated by the following example. In the *lacZ* gene in liver of the Muta™Mouse, the mutant frequency was 15- to 20-fold higher after exposure to MeIQx for 30 weeks than after just 10 days (189,193). This large increase in mutant frequency with chronic exposure to MeIQx occurred as the concentration of MeIQx–DNA adducts in liver

increased only 2-fold. Therefore, the likelihood of a mutagenic event increases with prolonged exposure to AIA–DNA adducts *in vivo*, apparently as ample time is provided for the fixation of mutations during cellular replication.

Tissue-specific factors, such as the rate of cell proliferation and cell death, play an important role in the mutagenicity of AIA–DNA adducts *in vivo*. Mutations in the *lacI* and *lacZ* genes of transgenic mice have also been shown to vary among tissue sites (187,188,190,191). In addition, the frequency of certain types of mutations was also different between organs indicating that multiple tissue-related factors potentially influence the mutagenic spectra induced by AIA–DNA adducts. In *lacI* transgenic mice on a diet of 0.03% MeIQ for up to 12 weeks, mutant frequency was highest in the colon, followed by the bone marrow, liver and forestomach; however, there was no increase in mutant frequency in heart, a tissue showing little proliferation (187). Over-expression of the *c-myc* oncogene has also been shown to increase the frequency of AIA-induced mutations in the *lacZ* reporter gene in liver of c-*myc*/*lacZ* double transgenic mice (189,193). The increased rate of hepatocellular proliferation associated with c-*myc* overexpression (200) may partly explain the higher mutant frequency in c-*myc* transgenic mice.

Whether *lacZ* or *lacI* reporter genes are general predictors of sites of chemical-induced carcinogenesis is still uncertain (190). Nevertheless, a recent study suggested that the mutational characteristics of individual AIAs in the *lacI* reporter gene of transgenic mice may represent the mutations induced by these AIAs in oncogenes and tumor suppressor genes involved in carcinogenesis. For example, PhIP was shown to induce a single-base deletion in the *lacI* gene in colon of transgenic mice that was characteristic of the single-base deletions found in the *Apc* gene in PhIP-induced rat colon tumors (192). The possible role of AIA–DNA adducts in carcinogenesis is discussed more fully below.

Implications of HCA–DNA adduct formation in animal models

Carcinogenesis: DNA adducts and target organ specificity

All of the AIAs demonstrated to be carcinogenic in rats and mice, including IQ, MeIQx, PhIP and MeIQ, form tissue DNA adducts in these species (79,89,90,118,121–141,201–212). In the liver, a target tissue for carcinogenesis by many AIAs (Table I), animal studies are consistent with the notion that AIA–DNA adduct levels are associated with carcinogenesis. Generally, the AIAs that induce hepatocellular carcinoma produce relatively high DNA adduct levels in the liver. IQ, but not MeIQx, is a potent hepatocarcinogen in cynomolgus monkeys (13,201,202). Accordingly, hepatic adduct levels in monkeys are ~50- to 100-fold higher with IQ than with MeIQx (83,120, 201). Both IQ and MeIQx are hepatocarcinogens in CDF_1 mice and Fischer-344 rats and both compounds produce relatively high hepatic DNA adduct levels (125,132,133,203–207). In contrast, PhIP, a compound that is not a hepatocarcinogen in adult mice or rats, forms relatively low hepatic DNA adduct levels in comparison with extrahepatic tissues, including the target organs mammary gland, prostate and colon (25,79,108,205,207,208).

Studies with MeIQx in rats are also consistent with the role of AIA–DNA adducts in hepatocarcinogenesis (22,132,133). MeIQx–DNA adduct levels in liver increased in a linear dose–response manner in rats fed various concentrations of MeIQx up to 400 p.p.m. (132,133). At 100, 200 and 400 p.p.m. MeIQx, the incidence of hepatocellular carcinoma was 0, 45 and 94%, respectively (22). These findings raise the possibility that a threshold dose of MeIQx, and perhaps a minimum level of MeIQx adducts, are needed for the induction of hepatocarcinogenesis. However, other factors associated with carcinogen exposure, such as tumor promotion, are also expected to play a role in hepatocarcinogenesis. It is noteworthy that MeIQx exposure is associated with the formation of 8-hydroxyguanine (213), a major species of oxidative DNA damage, which may also contribute to the carcinogenic effects of this compound.

Although the results from AIA–DNA adduct and carcinogenicity studies in liver support the role of AIA–DNA adducts in carcinogenesis, the finding that non-target tissues have relatively high AIA–DNA adduct levels belie a simple relationship between adducts and cancer (79,125,139,203–211). IQ–DNA adduct levels in Fischer-344 rats were reported to be highest in the liver, followed, in order, by the lungs, kidneys, stomach, colon, white blood cells (WBCs) and small intestine (205). Of these, only the liver, small intestine and colon are target organs in a carcinogenicity bioassay in Fischer-344 rats. The lungs, kidneys and stomach do not succumb to the carcinogenic effects of IQ, although adduct levels in these organs are higher than those in target tissues, including the colon and small intestine. As elevated AIA–DNA adduct levels are not observed in all target organs, it is possible that the threshold for the initiation of carcinogenesis by these adducts is unique among different tissues.

Neither the persistence of total AIA–DNA adducts nor the formation and persistence of specific AIA adducts appear to correlate with target organ specificity (121,122,133). In rats and monkeys, dG-C8–IQ, the principal IQ–DNA adduct formed *in vivo*, is removed more rapidly than dG-N^2–IQ in slowly dividing tissues, such as liver and kidney (121,122). In monkeys that develop hepatocellular carcinoma after chronic feeding with IQ, dG-N^2–IQ is the predominant adduct found in liver (122). However, this adduct is also the predominant adduct found in non-target tissues in monkeys, including kidney, pancreas and heart.

In summary, it is apparent that without metabolic activation and DNA adduct formation, AIAs would not be carcinogenic in animal models. However, since AIA–DNA adducts are found in both target and non-target tissues for AIA carcinogenesis, the relationship between AIA–DNA adduct formation and tumorigenesis is not a simple matter of cause and effect. In the subsequent section, the possible link between AIA–DNA adducts and carcinogenesis is further examined in a review of studies on mutations in specific oncogenes in AIA-induced tumors.

DNA adducts and oncogene activation in AIA-induced tumors

Specific mutations are critical for the activation of oncogenes and inactivation of tumor suppressor genes associated with carcinogenesis (214–216). Several studies have examined AIA-induced tumors for mutations in genes including p53, Ki-*ras* and Ha-*ras*, *Apc* and B-catenin (217–230). Among the mutations detected in these genes, guanine base mutations occurred with the highest frequency, suggestive of the involvement of AIA–DNA adducts in these genetic alterations. For example, mutations in either Ha-*ras* and Ki-*ras* were found in nine of nine Zymbal gland tumors induced by IQ (224). Eight of these mutations occurred at guanine bases in

codons 13 of H-*ras* or 12 and 13 of Ki-*ras*. Seven mutations were transversion mutations, characteristic of IQ-induced mutations *in vitro* (174). Only one IQ-induced Zymbal gland tumor carried an adenine mutation in codon 61 of Ha-*ras*.

Recently, colon tumors induced by IQ and PhIP were shown to harbor either mutations in *Apc* or B-catenin, providing evidence for a role of B-catenin/*Apc* pathways in the development of AIA-induced colon tumors (230). In addition, Kakiuchi *et al.* (229) showed that four of eight PhIP-induced colon tumors in F344 rats carried a 5′-GGGA-3′ to 5′-GGA-3′ frameshift mutation in the *Apc* gene. This mutation was also the most common of the frameshift mutations found in the *hprt* gene of Chinese hamster ovary cells exposed to PhIP (180). Interestingly, a guanine deletion in this and similar sequence contexts was observed in other *in vitro* mutagenicity assays with PhIP in the *hprt* gene of human lymphoblastoid cells, in the *supF* shuttle vector system and in the *dhfr* gene of Chinese hamster ovary cells (174–176), as well as in the *lacI* transgenic mouse model after PhIP treatment (192). These studies provide supporting evidence for a mutational fingerprint for PhIP–DNA adduct formation that involves a –1 frameshift hotspot in a run of guanine nucleotides adjacent to an adenine. The finding that this same mutation occurs in the *Apc* gene in rat colon tumors induced by PhIP (229,231) supports the importance of this specific PhIP–DNA adduct induced mutation in colon carcinogenesis. It also raises the possibility that this specific mutation in the *Apc* gene in human colorectal tumors may serve as a fingerprint for PhIP exposure and a means to assess the role of PhIP as an etiological factor in human colorectal cancer (180,214,229).

DNA adducts and cardiotoxicity

In both monkeys and rats fed IQ and PhIP, high levels of carcinogen–DNA adducts have been observed in the heart (126,139,209,232). Ultrastructural studies on the myocardium of monkeys undergoing carcinogenicity bioassay with IQ revealed myocyte degeneration with mitochondrial abnormalities (233). Similar changes were also observed in rats given IQ or PhIP (234,235). Both PhIP and IQ formed adducts in mitochondrial DNA (236), a factor that may contribute to mitochondrial damage. However, the role of adducts in AIA cardiotoxicity is not fully known. In isolated cardiomyocytes, *N*-acetylcysteine was shown to protect against *N*-hydroxy–IQ- and *N*-hydroxy–PhIP-induced cardiotoxicity, but the protective effect of *N*-acetylcysteine in isolated cardiomyocytes was not associated with a reduction in DNA adduct levels (237). It is possible that the reactive metabolites of IQ and PhIP may have other toxic effects on cardiomyocytes besides DNA adduct formation that are mitigated by *N*-acetylcysteine. Superoxide radicals have been suggested to be produced from AIA metabolism (238); whether these radicals play a role in the cardiotoxic properties of AIAs requires further investigation.

Inhibition of AIA–DNA adduct formation and chemoprevention of AIA-induced tumors

A number of cancer chemopreventive agents act by inhibiting the initiation phase of chemical carcinogenesis. One of the approaches in monitoring such inhibitory effects is by evaluating carcinogen–DNA adduct formation, and relating the extent of inhibition of this process to antimutagenic/anticarcinogenic effects of the agent. A variety of dietary components inhibit the mutagenicity of HCAs in the Ames *Salmonella* assay. These include hemin, chlorophyllin (CHL), retinol, fatty acids (arachidonic acid, oleic acid, linoleic acid, eicosapentaenoic acid, docosahexaenoic acid), flavonoids (morin, myricetin, quercetin, anthraflavic acid), polyphenols (ellagic acid, green tea polyphenols) and tryptamine (reviewed in refs 239,240). Human urine may also contain substances that inhibit HCA-induced bacterial mutagenicity (241); whether these substances are of dietary origin is not known.

In *in vivo* studies in rodents, a number of agents have been shown to inhibit AIA–DNA adduct formation in various organs, usually including the target organ (Table III) (242–256). The dietary agents studied most widely include CHL, conjugated linoleic acid (CLA) and indole-3-carbinol (I3C) (242–247). CHL is thought to act by non-covalent complexing with the AIA so that less is available for activation (242–245); it may also inhibit CYP1A2 (246). The mechanism by which CLA inhibits AIA–DNA adduct formation is not fully understood (248–250) whereas I3C appears to enhance AIA detoxification (247,254,255).

Whereas the chemopreventive properties of the inhibitors listed in Table III are known from studies in other model systems, the relationship between inhibition of AIA–DNA adducts and inhibition of AIA tumorigenesis has only been established for CHL as the inhibitor and IQ as the carcinogen (242,244,257). Phenethyl isothiocyanate, however, a well known experimental chemopreventive agent with other chemical carcinogens, did not inhibit PhIP–DNA adduct formation in liver and colon of male Swiss Albino mice (258).

With one exception (256), the dietary concentrations of the inhibitors listed in Table III were not toxic to the animals. In certain organs, or with certain protocols, however, both CHL and I3C may act as tumor promoters (257,259–261). Further studies are therefore needed on the mechanism of action of these agents and on their chemopreventive effects in AIA-induced tumorigenesis. In several studies, the relationship between inhibition of AIA–DNA adduct formation and inhibition of aberrant crypt foci formation, a putative pre-cancerous lesion in the colon, has already been established (247,249,254,255). In addition, several studies have been conducted on chemoprevention of AIA-induced tumors. Dietary CHL inhibited the multiplicity, but not the incidence, of PhIP-induced mammary adenocarcinomas in female F344 rats (262). The addition of lyophilized cultures of *Bifidobacterium longum* to the diet (0.5%) inhibits IQ-induced tumors in F344 rats (263). Dietary calcium inhibits PhIP-induced aberrant crypt foci formation in male F344 rats on low fat diets (264), whereas dietary oltipraz inhibits PhIP-induced lymphomas in male F344 rats (26). A number of antioxidants have been shown to possess anticarcinogenic properties against AIAs (265–267) but, with the exception of the studies on green and black tea (254), effects on DNA adduct formation were not evaluated in these studies.

AIA–DNA adducts and human cancer risk

Carcinogen–DNA adducts are regarded as biomarkers of potential mutagenic events and of cancer risk (31,268). Measurement of AIA–DNA adducts in tissues from humans eating customary diets, however, has proven to be difficult. For the purpose of biomonitoring and risk assessment, a satisfactory method to routinely measure AIA–DNA adducts in people has not yet been developed.

Several laboratories have attempted to measure AIA–DNA adducts in tissues or WBCs from humans eating customary

Table III. *In vivo* inhibition of AIA–DNA adduct formation in rodents

Inhibitor[a]	AIA[b]	Species (gender[c])	Organ	Reference
CHL	IQ	F344 rat (m)	Liver, small intestine, colon	242–244
CHL	PhIP	F344 rat (m)	Cecum, liver, heart	247
CLA	IQ	CDF_1 mouse (m,f)	Liver, lungs, colon, kidneys	248
CLA	IQ	F344 rat (m)	Colon	249
CLA	PhIP	F344 rat (f)	Liver, mammary gland	250
Menhaden oil	IQ	F344 rat (m)	Liver, lungs, stomach, kidneys	251
Menhaden oil	IQ	CDF_1 mouse (m)	Stomach, cecum, colon, kidneys	252
ω-3 Fatty acids	PhIP	CDF_1 mouse (m)	Liver, stomach, small intestine, cecum, spleen	253
I3C	PhIP	F344 rat (m)	Colon, cecum, heart, spleen	247
I3C	IQ	F344 rat (m)	Colon	254,255
I3C	PhIP	F344 rat (f)	Liver, colon, mammary gland	208
4-Ipomeanol	IQ	CDF_1 mouse (m)	Liver, lungs, stomach	256
Green tea	IQ	F344 rat (m)	Liver	254
Black tea	IQ	F344 rat (m)	Liver	254

[a]The inhibitor typically was provided in the diet (most of the time powdered AIN-76A diet). Green tea and black tea were provided as the sole source of drinking water.
[b]The AIA was provided in the diet, or given i.p. or p.o. (single dose or multiple doses).
[c]m, male; f, female.

diets. One study reported the detection of PhIP–DNA adducts in surgical samples of human colon by ^{32}P-post-labeling and gas chromatography–mass spectrometry (GC–MS) (210). Out of a total of 24 individual human tissue samples, including pancreas (n = 12), colon mucosa (n = 6) and urinary bladder epithelium (n = 6), only two of the colon samples had detectable DNA adducts (210). In another study (269), a total of 38 human tissues (colon, rectum, liver, kidney, pancreas, lung, heart), obtained from surgery or autopsy, were examined for the presence of dG-C8–MeIQx by ^{32}P-post-labeling. Only three specimens (one each of colon, rectum and kidney) were found to have detectable levels (1.8–14 adducts/10^{10} nucleotides).

Human WBCs are an easily accessible source of DNA for DNA adduct studies and potentially for biomonitoring of AIA exposure. However, in humans consuming meals with well-done meat, WBC AIA–DNA adducts are non-detectable by the ^{32}P-post-labeling method (P.T.Strickland and H.A.J.Schut, unpublished data). Studies in rats receiving daily doses of PhIP approximating the human daily intake (0.1 μg/kg) have shown that at this dose, or at doses that are even 10- or 100-fold higher, PhIP–DNA adducts are also non-detectable in tissues or WBCs when analyzed by ^{32}P-post-labeling (211). Therefore, for the purpose of evaluating the potentially genotoxic dose of AIA derived from the diet in humans, the likelihood of detecting AIA–DNA adducts in WBCs using established methods is low.

Until recently, one of the most sensitive methods for detecting DNA adducts was ^{32}P-post-labeling (270). This method has been applied to the detection of DNA adducts in situations where exposure to genotoxins is expected to be high, such as in smokers or in workers in certain occupational settings (268). The recent advent of the use of accelerator mass spectrometry (AMS) has made it possible to measure DNA adducts at levels as low as 1–10 adducts/10^{12} nucleotides (271–276), i.e. several orders of magnitude lower than the most sensitive version of the ^{32}P-post-labeling assay (270). Using AMS it has been possible to detect AIA–DNA adducts in tissues after dosing animals with amounts equivalent to human dietary exposures (273–275). Recently both MeIQx- and PhIP–DNA adducts have been detected in colon of humans given dietary equivalent levels of these AIAs labeled with ^{14}C (K.W.Turteltaub, personal communication). These studies indicate that at doses derived from the human diet, AIAs are potentially genotoxic and carcinogenic at specific target sites. In combination with the existing epidemiological evidence linking consumption of well-cooked meat with colorectal cancer risk (14,277–284), the results from AMS may eventually help to determine if human colorectal cancer risk is associated with AIA–DNA adduct formation.

Perspectives

There is considerable evidence to indicate that man is exposed to AIAs through the diet and is a species susceptible to the carcinogenic effects of AIAs (40,45,46,65,285–296). The unresolved question, however, is whether humans are exposed to levels of AIAs sufficient to play a significant role in human cancer incidence. The overall estimates of cancer risk from HCAs, calculated based on feeding studies in rodents (Table I), appear, so far, to be relatively small and insufficient to associate their presence directly with human cancer (14,297,298). However, there is increasing evidence to support that the risk of colorectal cancer and other cancers is higher in a subset of individuals who have particularly high intakes of well-done cooked meats likely to be rich in AIAs (279–284,299). Although there is wide interindividual variation in the capacity of human tissues to activate AIAs (45,83,293,300), in the case of colorectal cancer, risk appears to be especially elevated when high exposure is coupled with a strong capacity to metabolically activate the AIAs (283,284) which in turn implies the possibility of higher AIA–DNA adduct levels. The data summarized in this review support the notion that AIA–DNA adducts play a fundamental role in susceptibility to AIA carcinogenesis. As shown in animal studies, AIA–DNA adduct levels in target tissues are generally predictive of tumor incidence. Development of novel methods to detect AIA–DNA adducts in human tissues or the use of biomarkers of AIA–DNA adduct mediated genotoxic events is likely to provide further insight into the role of AIA–DNA adducts in carcinogenesis in humans.

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